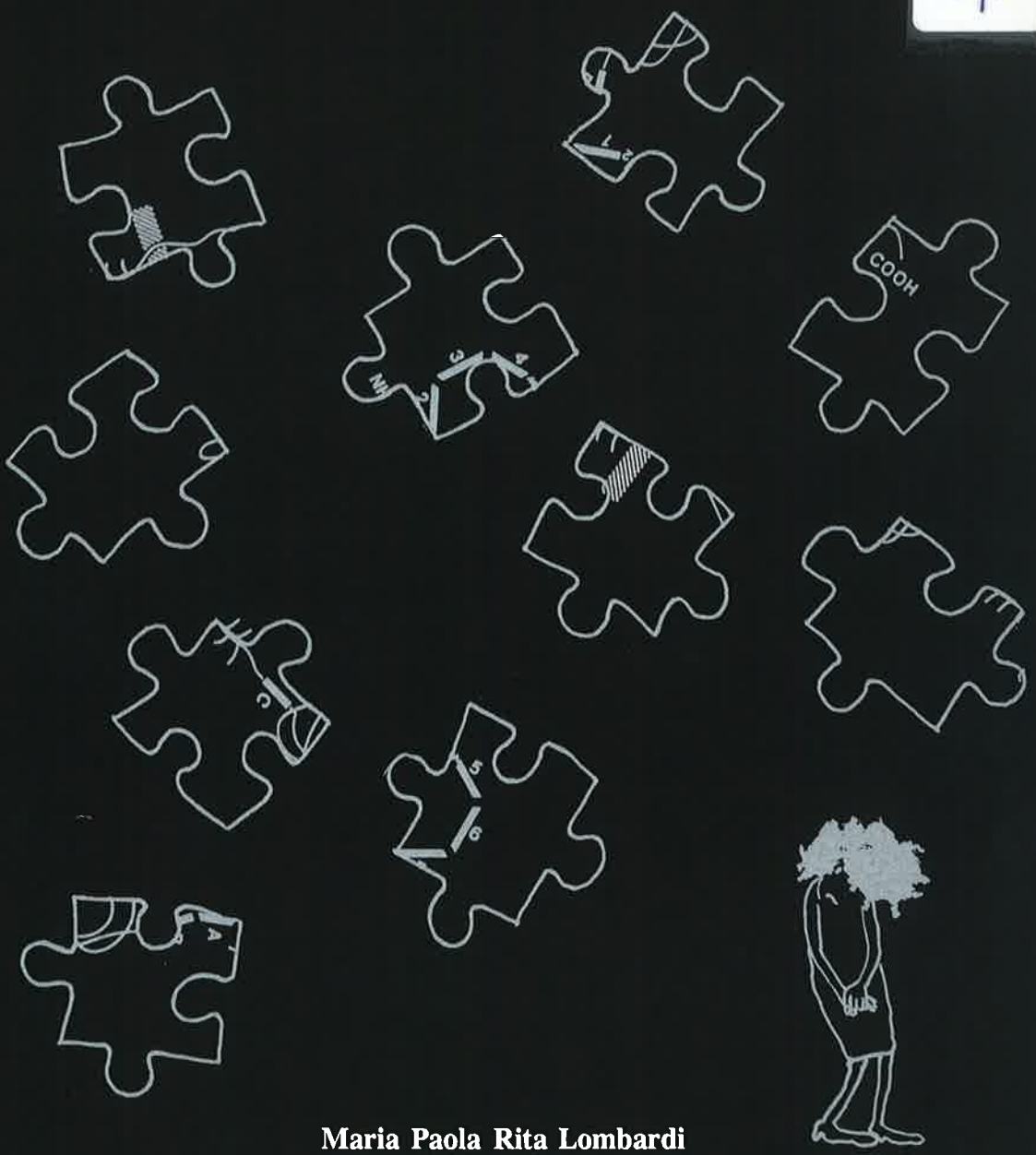
BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS OF THE LOW DENSITY LIPOPROTEIN RECEPTOR



Maria Paola Rita Lombardi

BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS OF THE LOW DENSITY LIPOPROTEIN RECEPTOR

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Lombardi, Maria Paola Rita

Biochemical and molecular genetic analysis of the low
density lipoprotein receptor / Maria Paola Rita Lombardi.
- [S.l. : s.n.]. - Ill.
Proefschrift Leiden. - Met lit. opg.
ISBN 90-5412-014-2 geb.
Trefw.: lipoproteïnen.

STELLINGEN

1. For screening of LDL receptor gene mutations, denaturing gradient gel electrophoresis is a more sensitive, environment-friendly technique as compared to single strand conformation polymorphism.
(this thesis)

2. It has not been proven that lowering of serum cholesterol levels induces aggressive behaviour.
H. Engelberg (1992) The Lancet 339: 727-729

3. The observation that a sequence present in the promoter region of a given gene can mediate a retinoic acid response in transfection experiments does not necessarily imply that this element is retinoic acid-responsive under *in vivo* conditions.
M. Petkovich (1992) Annu. Rev. Nutr. 12: 443-771

4. Although a "founder" effect through migration and geographical or cultural isolation seems unlikely in most parts of Europe, the frequency of some mutations still may be high in certain local areas.
Webb et al. (1992) J. Lipid Res. 33: 689-698; Top et al. (1990) Atherosclerosis 83: 127-136 and this thesis

5. The suggested relationship between temperament (i.e. activity, sociability, and emotionality) and apoE phenotypes indicates that there is still much to learn about genetic factors and behaviour.
Keltikangas-Järvinen et al. (1993) Psychosomatic Medicine 55: 155-163

6. The statement "Anything under the moon and the sun can influence the polymerase chain reaction (PCR) specificity" proves wrong those who think that PCR is easy.
J.J. Sninsky, personal communication

7. The way the general press reported the genetic study conducted by D.H. Hamer involving the existence of a gene on the X chromosome that induces male sexual orientation emphasizes that there is a long way to go before public understanding of science is reached.
Hamer et al. (1993) Science 261: 321-327

8. The recent finding that wine's therapeutic benefits are not due to the alcohol but to phenolic compounds, acting as anti-oxidants and performing an "aspirin-like" function, will disappoint those who had one too many for medical reasons.
Science (1993) 260: 1239

9. Functionally, splice site sequences form part of the coding sequence.
(this thesis)

10. A chocoholic is a socially acceptable addict.
B. Max (1992) TiPS 13: 341-345

11. For cooking or conducting an experiment, a good recipe is no guarantee of success.

12. De laatste loodjes hebben dezelfde dichtheid.

Oegstgeest, 13 October 1993 M. Paola Lombardi

BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS OF THE LOW DENSITY LIPOPROTEIN RECEPTOR

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op woensdag 13 oktober 1993
te klokke 15.15 uur

door

Maria Paola Rita Lombardi

geboren te Milaan in 1960

Promotiecommissie:

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	Prof.dr. A.L. Catapano (Universiteit van Milaan, Italië)
	Prof.dr. G.J. van Ommen

The studies presented in this thesis were performed at the IVVO-TNO, Gaubius Laboratory and at the former Gaubius Institute TNO in collaboration with the MGC-Department of Human Genetics, Leiden and, partly, at the Institute of Pharmacological Sciences, University of Milan. The studies were financially supported by the Netherlands Heart Foundation (project # 89.057).
Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged. Additional support was obtained from the IVVO-TNO, Gaubius Laboratory, Leiden.
Cover design: Monique Mulder

Aan mijn ouders
Voor Sergio

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1 Lipoprotein metabolism

Introduction

Cholesterol is required by all animal cells for the growth and maintenance of the bilaminar cell membranes. In general, the requirement for cholesterol is much greater in cells that are dividing or growing rapidly than in those that are in a resting state. Cholesterol is also required as a precursor for bile acid synthesis by the liver and for steroid hormones by the gonads and adrenal cortex. Triglycerides are used as a source of energy in the muscles and for storage in the adipose tissue. Both cholesterol and triglycerides are highly hydrophobic and, therefore, not soluble in an aqueous environment such as blood. In the blood-stream, they are transported in the form of complexes of lipids and proteins, called "lipoproteins". These are spherical particles which contain both a central core of non-polar lipids, primarily triglycerides and cholesterol esters, and a surface monolayer of polar lipids, primarily free cholesterol, phospholipids and apolipoproteins.

The lipoproteins have been subdivided into five major classes, according to their buoyant density: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). These lipoproteins, as well as varying in density, differ in size, composition and electrophoretic mobility. Their properties are summarized in Table 1 (for reviews on lipoproteins and apolipoproteins see Havel and Kane, 1989; Mahley et al., 1984; Gotto et al., 1986; Breslow, 1988, 1991).

The lipoprotein metabolism can be subdivided in three different parts: (*i*) the exogenous lipid transport, (*ii*) the endogenous lipid transport and (*iii*) the reverse cholesterol transport (for reviews see Havel and Kane, 1989; Breslow, 1989; Gibbons, 1990; Mahley and Hussain, 1991).

The exogenous lipid transport

In the exogenous lipid transport (Fig. 1) dietary triglycerides and cholesterol are absorbed and packaged into large triglyceride-rich particles called chylomicrons in the intestinal epithelial cells. The major apolipoprotein (abbreviated as apo) components are apo A1, apoA4 and the intestinal form of apoB (B48). After entering the blood-stream via the lymph, chylomicrons acquire C apolipoproteins and apoE by transfer from HDL. ApoC2 is a

Table 1. Biophysical and chemical properties of human plasma lipoproteins.

Lipoprotein class	Source	Biophysical properties: Diameter (nm)	Electrophoretic mobility	Density (g/ml)	S_f	Molecular weight
Chylomicrons	Intestine	75-1200	Origin	< 0.96	> 400	50-1,000 x 10^6
VLDL	Liver + intestine	30-80	pre-β	0.96-1.006	20-400	10-80 x 10^6
IDL	VLDL + chylomicrons	25-35	slow pre-β	1.006-1.019	12-20	5-10 x 10^6
LDL	VLDL	18-25	β	1.019-1.063	2-12	2,300,000
HDL	Liver + intestine	5-12	α	1.063-1.210		175-360,000 x 10^3

	Chemical properties: Protein % weight	Total lipid % weight	Triglycerides (% of total lipid)	Phospholipid (% of total lipid)	Cholesterol ester (% of total lipid)	Free cholesterol (% of total lipid)	Major apolipoproteins
Chylomicrons	1-2	98-99	88	8	3	1	A1,A4,B48,C1,C3,E
VLDL	6-10	90-94	56	20	15	8	B100,C1,C2,C3,E
IDL	11	89	29	26	34	9	B100,E
LDL	21	79	13	28	48	10	B100
HDL	45-55	45-55	15	45	30	10	A1,A2,E

cofactor for the enzyme lipoprotein lipase (LPL). This enzyme resides on the capillary endothelium and hydrolyzes most of the core triglycerides. Concomitantly, excess surface components (phospholipids and apoA1) are transferred to HDL, while apoE is acquired from HDL. The residual particles, now called chylomicron remnants, are enriched in apoE and cholesterol. These remnant particles are efficiently cleared by hepatic lipoprotein receptors, which recognize apoE on the remnant surface. The identity of a specific chylomicron remnant receptor is still under debate. Studies by Hertz et al. (1988) and Kowal et al. (1989) have indicated the low density lipoprotein receptor-related protein (LRP) as the putative chylomicron remnant receptor. More recently, evidence has been provided that the LRP might be a multifunctional receptor, capable of binding other molecules such as α_2-macroglobulin (Strickland et al., 1990) and tissue-type plasminogen activator (Bu et al., 1992) as well as chylomicron remnants. Recently, a putative recognition site for β-VLDL (referred to as a remnant receptor) with different distinctive properties from that of LRP/α_2-macroglobulin receptor has been identified in rat parenchymal liver cells (Van Dijk et al., 1992).

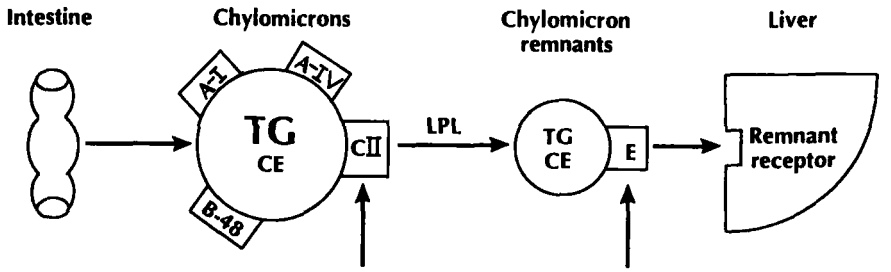

Figure 1. Exogenous pathway (from J.L. Breslow, J. Clin. Invest. 1989; 84: 373-380, reprinted with permission).

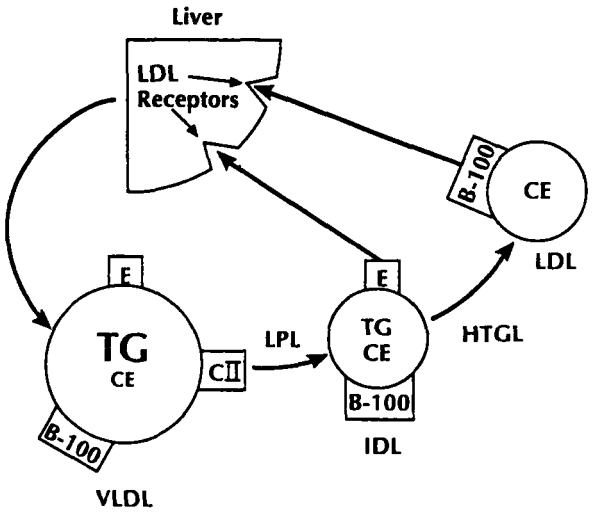

Figure 2. Endogenous pathway (from J.L. Breslow, J. Clin. Invest. 1989; 84: 373-380, reprinted with permission).

Once taken up, the remnants are endocytosed and catabolized in the lysosomes, from which cholesterol can enter different metabolic pathways in hepatocytes, including VLDL synthesis and conversion into bile acids.

The endogenous lipid transport

The endogenous lipid transport (Fig. 2) involves the production and secretion of VLDL by the liver. VLDL provides a pathway for the export of triglycerides, either derived from lipogenesis, carbohydrates and plasma free fatty acids, or taken up from chylomicron remnants. VLDL, similar to chylomicrons, are triglyceride-rich, cholesterol containing particles with apoB100, apoE and apoC as surface components. As with chylomicrons, most VLDL triglycerides are hydrolyzed in extrahepatic tissues by LPL to yield remnant particles or IDL, which are relatively enriched in cholesterol and apoE. Low density lipoprotein (LDL) receptors on hepatocytes recognize apoE on VLDL remnants and mediate the endocytosis of a substantial fraction of these particles. Some, however, are further processed, presumably by a lipase on hepatic cell surfaces (HTGL), to produce LDL particles, which mainly contain cholesterol as the lipid component and apoB100 as the sole apolipoprotein. LDL can be taken up by the liver *via* LDL receptors, which recognize apoB100 and account for approximately 70% of LDL removal from the circulation. Unlike its remnant precursor, which has a life span of few hours, LDL circulates in the blood for days, due to the lower affinity of apoB100 for the LDL receptor as compared to apoE. Although most of the LDL clearance and catabolism occurs in the liver, various extra-hepatic tissues express LDL receptor activity, e.g. muscle, adipose tissue and adrenal glands.

A second pathway involved in the clearance of LDL from the circulation is the LDL receptor-independent pathway, which may be mediated by the scavenger receptor, that is present in cells of the reticulo-endothelial system, like macrophages (Smedsrød et al., 1990; Emi et al., 1993). This receptor, unlike the LDL receptor, is not subjected to feed-back regulation and does not recognize native LDL, but avidly takes up LDL which has undergone oxidative modification, a process which has been shown to take place by simply incubating LDL for a number of hours with monolayers of endothelial cells, smooth muscle cells, monocytes or macrophages (Steinberg, 1993). Strong evidence has been recently provided (Steinberg, 1993) showing the correlation between (*i*) the formation of oxidized LDL and the scavenger receptor activity and (*ii*) the formation of "foam cells" in the intima of the blood vessels. This formation is suggested be one of the initial steps in the generation of atherosclerotic lesions (Brown and Goldstein, 1983).

Reverse cholesterol transport

Reverse cholesterol transport (Fig. 3) provides a means for cholesterol, which cannot be metabolized by peripheral tissues, to be transported from these tissues back to the liver. Nascent HDL, discoidal particles principally containing phospholipids and apoA1, are produced by the liver. These particles attract additional surface components formed during the lipolysis of the triglyceride-rich lipoproteins, chylomicrons and VLDL, and can

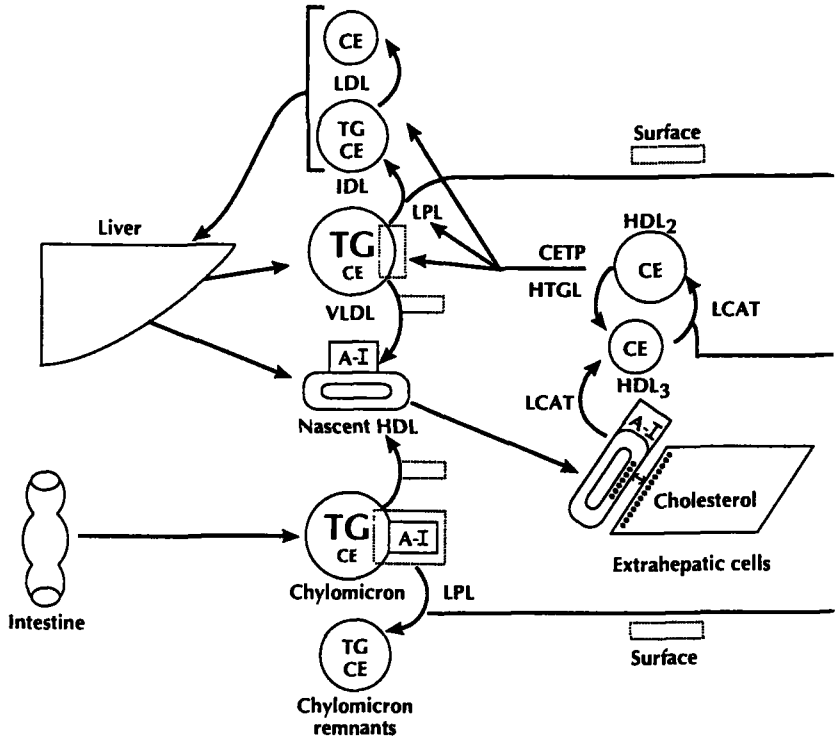

Figure 3. Reverse cholesterol transport pathway (from J.L. Breslow, J. Clin. Invest. 1989; 84: 373-380, reprinted with permission).

subsequently take up free cholesterol from extrahepatic cells. This cholesterol is esterified in plasma by the enzyme lecithin cholesterol acyltransferase (LCAT), which uses apoA1 as a cofactor. The so-produced cholesterol esters enter the core of HDL, changing the shape of the particle into a sphere, known as HDL_3 or small HDL. The additional action of LCAT further enriches the HDL particles with cholesterol esters forming large HDL_2 particles. The cholesterol ester transfer protein (CETP) exerts the opposite action: it reconverts HDL_2 into HDL_3 by promoting the transport of cholesterol ester from HDL_2 to the apoB-containing particles (chylomicrons, chylomicron remnants, VLDL, IDL and LDL). This process allows the uptake of cholesterol esters by the liver *via* the LDL receptor pathway and subsequent elimination of cholesterol from the body. Part of the HDL_2 particles have been suggested to be directly removed from the circulation *via* a receptor-mediated pathway, which might use apoA1 as the ligand. However, a HDL receptor pathway has not been fully elucidated yet.

The coordinated regulation of the lipoprotein transport, through the action of apolipoproteins, enzymes and specific receptors, determines the plasma lipid concentrations. Environmental and genetic factors that alter the lipoprotein transport often lead to accumulation of specific plasma lipoproteins and, consequently, to hyperlipidemia (Breslow, 1988, 1989; Schaefer et al., 1988). In this thesis studies are presented focusing on one of the genetic lipoprotein disorders that have been reported: familial hypercholesterolemia.

1.2 Familial hypercholesterolemia: Clinical aspects

Familial hypercholesterolemia (FH) was described for the first time more than a century ago as a disease of the skin (Fagge, 1873). It was 60 years later that the association between the presence of hypercholesterolemia and xanthomas, and the incidence of premature atherosclerosis was established (Müller, 1938). In the following decades, family studies revealed the hereditary basis of FH (Wilkinson et al., 1948) and the autosomal dominant mode of inheritance (Kachandurian, 1964). At the same time it became evident that a selective increase in LDL concentration is responsible for the hypercholesterolemia (Fredrickson et al., 1967). In the 1970s the work of Brown and Goldstein revealed that the primary abnormality underlying FH is a defective LDL-receptor function (Brown and Goldstein, 1974a,b, 1976) due to mutations in the LDL receptor gene (Brown and Goldstein, 1986).

Genetics of FH

FH is an autosomal disorder, with a dominant mode of inheritance, characterized by an elevation in plasma LDL cholesterol (Goldstein and Brown, 1989). FH occurs in two forms. The heterozygous form is rather common, affecting approximately one person in every five hundred. FH heterozygotes, who inherit one mutant LDL receptor allele, manifest a 2- to 3-fold elevation in plasma LDL-cholesterol and typically develop premature coronary heart disease after 35 years of age. FH homozygotes, who inherit two mutant receptor alleles, are rare (1 in 10^6 individuals) and more severely affected. Their plasma cholesterol levels are increased 6- to 8-fold and they often die before the age of 20 of myocardial infarction. It should be noted that the term FH homozygote refers to individuals who inherit two identical mutant alleles (true homozygotes) as well as to individuals who inherit two different mutant alleles (compound heterozygotes). The latter group represents the vast majority of the FH homozygous population (Hobbs et al., 1992).

Although in most populations the frequency of heterozygous FH is approximately 0.2%, in some populations, including Afrikaners, French Canadians, Ashkenazi Jews of Lithuanian origin, Christian Lebanese, Druze, Sephratic Jews and Finns, a much higher prevalence has been recorded. As an example, among the Afrikaners the prevalence of heterozygous FH is as high as 1 in 100 (Gevers et al., 1987), while among the Lebanese population the

prevalence is about 1 in 170 (Lehrman et al., 1987a). This phenomenon has been attributed to a founder effect: a small number of mutations predominates in small populations, which for geographical, historical or religious reasons have been genetically isolated through the centuries. Table 2 summarizes the frequency of the mutations in the populations in which a founder effect exists.

Clinical features and diagnosis of FH

The most prominent sign and preliminary diagnostic marker of FH is a raised concentration of plasma LDL cholesterol, above 5 mmol/l (Thompson et al., 1989a), while the other lipid parameters remain in the normal range. Two factors play a major role in determining the extent of the increase of LDL cholesterol in FH: obviously, the first factor depends on whether the disease is in the heterozygous or in the homozygous form, and the second depends on the genetic defect causing the disease and on the severity of disruption of the LDL receptor function. In homozygotes, phenotypic expression of the disorder is dominated by genotypic variation at the LDL-receptor gene locus (Sprecher et al., 1985; Thompson, 1989b), while other factors, like gender, have little influence. In contrast, phenotypic variation in heterozygous is influenced not only by the nature of the underlying gene defect but also by age, gender, diet (Thompson et al., 1989b) as well as by the genetic polymorphism of the ligands for the LDL receptor, i.e., apolipoproteins E (Eto et al., 1988; Utermann, 1987; Gylling et al., 1991) and B (Aalto-Setälä et al., 1989; Gylling et al., 1991). An additional genetic factor, apart from the nature of the LDL receptor gene mutation, that might contribute to the variation of the phenotypic expression of heterozygous FH is a putative "cholesterol-lowering gene" that, in one family, has been found to normalize serum cholesterol levels of the known carriers of the LDL receptor gene mutation (Hobbs et al., 1989).

A consequence of the increased plasma LDL cholesterol level is cholesterol deposition in the skin (xanthelasma), tendons (xanthomas) and coronary arteries (atherosclerosis). Tendon xanthomas are the clinical hallmark of heterozygous FH, being frequently the first clinical sign (Fig. 4), while homozygous FH often have xanthomas already at birth (Goldstein and Brown, 1989). In heterozygous FH xanthomas represent an age-related phenomenon: they appear only in 7% of cases below the age of 20, whereas 75% of the heterozygous FH individuals around the age of 40 have xanthomas (Goldstein and Brown, 1989; Beaumont et al., 1976).

The elevation of plasma cholesterol results in premature atherosclerosis. In FH heterozygotes the mean age of onset of coronary artery disease (CAD) is above 40 years in males and above 50 years in females (Slack, 1969). In a large population sample, no cases of CAD were observed in patients without tendon xanthomas (Gagne et al., 1979). From data collected by several different groups in large population studies it has been estimated that the risk for myocardial infarction before the age of 60 is 75% for males and 45% for females (Goldstein and Brown, 1989). As mentioned before, homozygous FH rarely reach the age of 30, with no difference in the age of onset and prevalence of CAD between males and

Table 2. LDL receptor mutations in inbred populations (from Hobbs et al., Human Mutation, 1992, 1: 445-466, reprinted with permission).

Ethnic population	Geographic location	Frequency of FH heterozygotes in population	Mutant allele	Proportion of FH heterozygotes with mutant allele (%)	References
Ashkenazi Jews of Lithuanian ancestry	South Africa	1/67	*FH Lithuania*	80	Hobbs et al., 1990; Seftel et al., 1989; Meiner et al., 1991
	Israel	not known	*FH Lithuania*	33	
French Canadians	Quebec Province, Canada	1/270	*FH French Canadian-1*	60	Hobbs et al., 1987, Ma et al., 1989; Leitersdorf et al., 1990; Moorjani et al., 1989
			FH French Canadian-2	5	
			FH French Canadian-3	2	
			FH French Canadian-4	7	
			FH French Canadian-5	5	
Afrikaners	South Africa	1/100	*FH Afrikaner-1*	65-70	Leitersdorf et al., 1989a; Kotze et al., 1991 Van Roggen et al., 1991
			FH Afrikaner-2	20-25	
			FH Afrikaner-3	5-10	
Christian Lebanese	Lebanon and Syria	1/170	*FH Lebanese*	~ 100	Lehrman et al., 1987a; Slack, 1979
Finnish	Finland	~ 1/500	*FH Helsinki*	30-40	Aalto-Setälä et al., 1992; Koivisto et al., 1992
			FH Karelia	34	

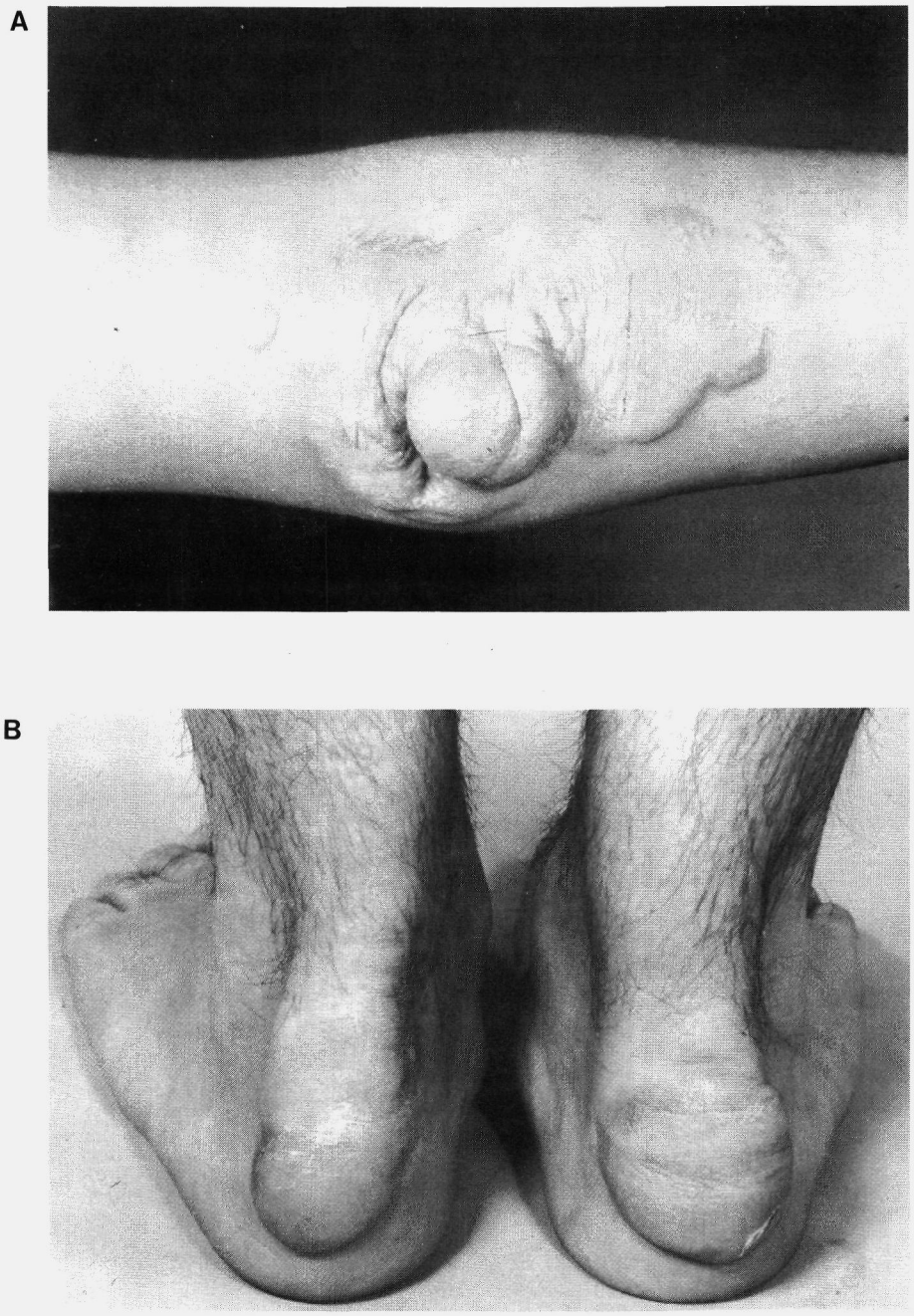

Figure 4. Clinical signs of FH. Xanthomas at the extendor tendon of the elbow (A) and at the Achilles tendon (B).

females.

Treatment of heterozygous FH is directed to decrease the LDL plasma cholesterol level by increasing the number of functional receptors. This result has been obtained using two different approaches: (*i*) by inhibiting HMG-CoA reductase, the rate limiting enzyme in the endogenous cholesterol synthesis, so that cells depend on an exogenous source of cholesterol or (*ii*) by using bile acids sequestrants, which prevent the bile acids to be reabsorbed and, consequently, increase the demand for cholesterol in the liver. Both pharmacological treatments are quite effective, leading to a reduction up to 15-20% of the plasma cholesterol level or even more, when they are used in combination (Goldstein and Brown, 1989).

FH homozygotes do not respond to pharmacological treatment, since they do not possess any functional receptor. Therefore, more drastic measures are required, such as the selective removal of plasma LDL (LDL-apheresis) or, ultimately, liver transplantation. The application of gene therapy is still in a preliminary phase of experimentation (Hofmann et al., 1988; Dodet, 1993; Dzau et al., 1993). A successful attempt to correct by gene transfer the genetic defect in hepatocytes from Watanabe heritable hyperlipidemic (WHHL) rabbits (Wilson et al., 1988) indicates that, in a near future, gene therapy might prove to be the best option for the treatment of homozygous FH (Williamson, 1993).

1.3 LDL receptor-mediated endocytosis

The evidence that LDL is cleared from the blood-stream through a specific, receptor-mediated pathway, came from the pioneering work of Goldstein and Brown on the binding of ^{125}I-LDL to normal and familial hypercholesterolemic (FH) homozygous fibroblasts (Goldstein and Brown, 1974). In 1976 Anderson, using LDL-ferritin complexes, discovered that the receptors are clustered in specialized areas of the cell surface, called coated pits (Anderson et al., 1976). A schematic representation of the LDL receptor endocytotic pathway is shown in Fig. 5.

Within 1-3 minutes of their formation, the pits invaginate into the cytoplasm and are pinched off from the plasma membrane to form coated vesicles, enclosing any LDL bound to a receptor. The coat is removed from the cytoplasmic face of the vesicle, either during invagination of the pits (Willingham and Pastan, 1980), or shortly thereafter (Anderson et al., 1977), to form uncoated vesicles called endocytic vesicles or receptosomes. These endocytic vesicles become larger possibly by fusing with each other. Due to the activity of an adenosine triphosphate-driven proton pump in the membrane, the pH of the endosomes decreases and, when it falls below 6.5, the LDL dissociates from the receptor. Goldstein et al. (1979) observed that the number of receptors on the surface of fibroblasts declines by only 50% in 20-25 hours, when the synthesis of new receptors is inhibited by cycloheximide. Since no reservoir of receptors is present in fibroblasts (Basu et al., 1978), each LDL receptor must return to the cell surface several times before it is degraded (Basu et al.,

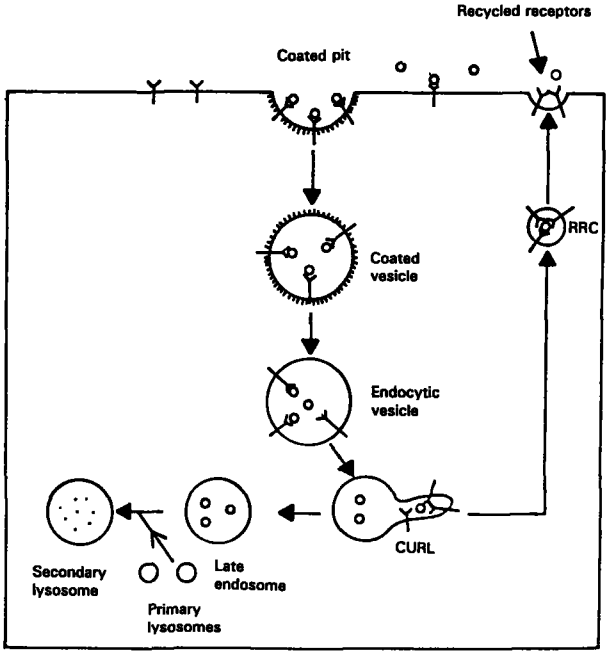

Figure 5. Endocytotic pathway of the LDL receptor protein (from N. Myant, In: Cholesterol metabolism, LDL and the LDL receptor, 1990, reprinted with permission).

1981), hence they must leave the endocytic vesicle before it fuses with a lysosome. The work of Geuze et al. (1983) clarified how the ligand and the receptor dissociate during their endocytotic pathway. They showed that the receptors and their ligands dissociate in vesicles with long tubular extensions, called "compartment of uncoupling of receptor and ligand" (CURL). The receptors are confined to the membrane of the extensions, while the ligand remains in the lumen of the vesicle. These tubular extensions are pinched off, forming the so-called recycling endosomes or receptor recycling compartment (RRC), and are transported to the plasma membrane, while the vesicles, now denominated late endosomes, fuse with the lysosomes. It has been noted that the recycling pathway of the receptors via the CURL does not necessarily take place after the dissociation of the ligand from the receptor but may also occur while the receptor-ligand complex is still intact (Gavigan et al., 1988). This might explain how retroendocytosis occurs. It has been shown by Aulinskas et al. (1985) that about 10% of the endocytosed LDL is excreted intact into the extracellular medium. This is suggested to be due to the trapping of LDL in the tubular extension of CURL, so that part of the internalized LDL escapes degradation and is routed back to the cell membrane together with the receptor. Each LDL receptor makes a round trip every 10 minutes, whether or not it is occupied with LDL. Since the estimated life-span of the receptor is 25 hours, each receptor recycles about 150 times (Goldstein et al., 1985).

Although the endocytotic pathway of internalized particles has been largely delineated, it still remains unclear how internalized molecules are transferred from the sorting endosomes, or CURL, to the late endosomes. Two mechanisms have been suggested (Helenius et al., 1983): (*i*) vesicular carriers could bud off from the sorting endosomes and deliver the lysosomally targeted molecules to the late endosomes and (*ii*) the sorting endosome itself could "mature" with the internalized molecules. Although in the recent years evidence has been provided supporting both models, the theory of a single step of maturation rather than repeated budding off of transport vesicles is gaining credit (Murphy, 1991; Stoorvogel et al., 1991).

1.4 LDL receptor activity in relation to cellular cholesterol homeostasis

When the membranes of the late endosomes and lysosomes fuse, the protein component of LDL is broken down to amino acids and the cholesterol esters are hydrolyzed by an acid lipase, liberating free cholesterol.

Two lines of evidence demonstrate the involvement of lysosomal hydrolases in LDL degradation. Firstly, the use of chloroquine or other ionophores, which raise the pH of the endocytotic vesicles, leads to an accumulation of intact particles within the secondary lysosomes (Goldstein and Brown, 1977). Secondly, in patients with monogenic disorders characterized by a specific deficiency of a lysosomal acid lipase (Wolman's disease and cholesterol-ester storage disease), the protein component of LDL is normally degraded, while the cholesterol esters accumulate intralysosomally.

The free cholesterol released during LDL catabolism is partly incorporated into newly synthesized surface membranes, especially in non-confluent cells. In specialized tissues, this cholesterol exerts other functions, for example in the adrenal gland and in the ovary it can be converted into steroid hormones, cortisol and estradiol, respectively, and in the liver it is transformed into bile acids and is used for VLDL synthesis. However, the leading role of cholesterol liberated from LDL is to control cellular cholesterol metabolism, acting at three different levels (Fig. 6). Firstly, it suppresses the synthesis of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, by inhibiting HMG-CoA reductase gene transcription and accelerating the degradation of the enzyme protein (Luskey et al., 1983; Gil et al., 1985). This leaves the cells dependent on an exogenous source of cholesterol, namely the one supplied by the receptor-mediated uptake of LDL. Secondly, free cholesterol stimulates the enzyme acyl CoA: cholesterol acyl transferase (ACAT), which activates the esterification of free cholesterol, so that excess cholesterol can be stored in the cytoplasm as cholesterol ester droplets. The third and most significant effect is the activation of a feedback mechanism that reduces the number of receptors expressed on the cell surface (Brown and Goldstein, 1975) (Fig. 6). This effect is entirely mediated through a decrease in the rate of synthesis, by lowering the concentration

of receptor messenger RNA, without any change in the rate of catabolism of the receptor protein.

The coordinated regulation of these three mechanisms provides the cells with sufficient cholesterol for metabolic need, but it prevents cholesterol overaccumulation.

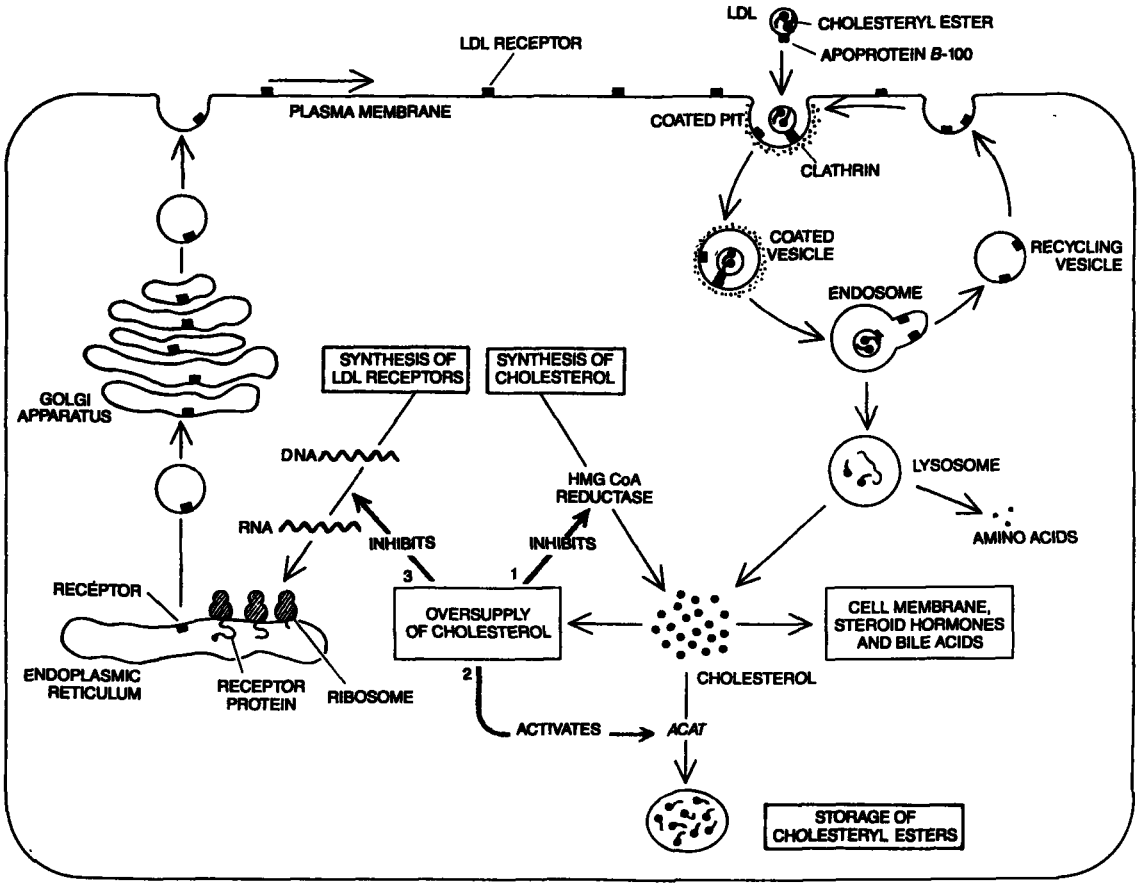

Figure 6. Regulatory and biosynthetic pathway of the LDL receptor protein (from Brown and Goldstein, Scientific American, 1984, 52-60, reprinted with permission).

LDL receptors in non-fibroblast cells

After the characterization of the LDL receptor in human skin fibroblasts, various methods were developed and used to establish the presence of LDL receptor activity in other cell lines and tissues. In this respect, a major step forward was made when the polyclonal (Beisiegel et al., 1981a) and monoclonal (Beisiegel et al., 1981b) LDL-receptor antibodies became available. Since then, using binding and competition studies, ligand- and immunoblotting

techniques, LDL receptor activity has been demonstrated in smooth muscle cells, endothelial cells, monocyte-macrophages, cells of the adrenal cortex and ovary, lymphocytes, adipocytes and hepatocytes (of many different species), as well as in subcellular membranes prepared from fresh tissues including liver, adrenal cortex, ovary, small intestine and kidney (Kovanen et al., 1979).

Although the LDL receptor protein in different tissues is identical, being encoded by the same gene, there are aspects related to the function and regulation of the receptor expressed in certain specialized cells, which differ from those described in fibroblasts. In this respect, particular attention has been devoted to the LDL receptors expressed in liver cells. Considering that the liver is the organ which mainly contributes to LDL catabolism, it is conceivable that differences in the function and regulation of the hepatic receptor will have a major impact on the overall cellular cholesterol metabolism.

Hepatic LDL receptors

LDL receptor activity was detected in human liver cell membranes (Hoeg et al., 1984; Soutar et al., 1986) as well as in freshly isolated human hepatocytes (Havekes et al., 1986a). Electron microscopic studies indicate that LDL can be taken up in the liver to a variable extent by hepatocytes, while a more constant contribution from non-parenchymal cells is noticed (Kleinherenbrink-Stins et al., 1991). In contrast with fibroblasts, the LDL receptors are distributed diffusely on the cell surface of hepatocytes, with no clustering in the coated pits (Havel and Hamilton, 1988). However, the ligand-receptor complexes do indeed move to the pits prior to internalization (Pathak et al., 1990). The effect of this difference in the initial step of the endocytotic pathway on the intracellular processing of LDL has not been clarified yet.

Hepatocytes are exposed to a concentration of LDL similar to that of plasma. Therefore, according to the fibroblast model, the LDL receptor activity in hepatocytes was expected to be almost completely suppressed at normal LDL concentrations. By contrast, several studies performed with hepatocytes in culture (Edge et al., 1986; Salter et al., 1987), freshly isolated human hepatocytes (Havekes et al., 1986a) and HepG2 cells (Havekes et al., 1986b) have shown that the LDL receptor expression is only weakly down-regulated in the presence of concentrations of LDL that abolish the receptor activity in fibroblasts. As a possible explanation, it has been suggested that the cholesterol liberated by LDL hydrolysis is directed to a non-regulatory (cholesterol esters) pool and/or to an extracellular acceptor of cholesterol, rather than to the regulatory free cholesterol pool (Havekes et al., 1987).

1.5 The LDL receptor protein

Structure and biosynthesis

The full-length human LDL receptor cDNA encodes a protein of 860 amino acids, which

includes a stretch of 21 amino acids, representing a *N*-terminal signal sequence, analogous to signal sequences in many membrane proteins (Blobel, 1980; Yamamoto et al., 1984). This directs the synthesis of the receptor in the endoplasmic reticulum and is cleaved from the receptor immediately after it is translated, leaving a receptor protein of 839 amino acids. The mature receptor is oriented with its *N*-terminal on the outside of the plasma membrane (Schneider et al., 1983) and the *C*-terminal sequence projected into the cytoplasm (Russell et al., 1984). The LDL receptor is a cell-surface glycoprotein that contains one or two asparagine-linked (*N*-linked) oligosaccharide chains and approximately 18 serine/threonine-linked (*O*-linked) oligosaccharide chains, most of which are clustered in a specific region of the molecule (Cummings et al., 1983).

Pulse-chase experiments showed that, when human fibroblasts are incubated in the presence of [^{35}S]-methionine and subsequently immunoprecipitated with an anti-LDL receptor monoclonal antibody (Beisiegel et al., 1981b), the LDL receptor is synthesized in the rough endoplasmic reticulum as a precursor molecule with a molecular weight of approximately 120 kD. Within 15-30 minutes, the precursor receptor starts to be converted into a mature form with a molecular weight of approximately 160 kD and within 1 hour the conversion is virtually complete (Tolleshaug et al., 1982). The work of Cummings et al. (1983) and Davis et al. (1986a) helped to elucidate the major events occurring during the conversion of the receptor protein from the precursor to the mature form. By using glycosylation inhibitors (tunicamycin) and enzymes which selectively cleave the *N*- or *O*-linked sugars (*N*- and *O*-glycosidases) they showed that the 120 kD precursor contains only one or two *N*-linked high mannose units, which are sensitive to the activity of endoglycosidase-H, and the core sugar (*N*-acetylgalactosamine) of the *O*-linked sugars. The receptor protein then moves to the Golgi apparatus, where the high mannose *O*-linked chains are converted to the endoglycosidase-resistant form and each *O*-linked chain is elongated by the addition of one galactose and two sialic acid residues. Apparently, the increase in molecular weight of the mature protein is entirely due to the elongation of the sugar chains in the Golgi apparatus. Since the amount of carbohydrates added is not sufficient to account for an increase of 40 kD in the molecular mass, it has been suggested that the elongation of the clustered *O*-chains produces a change of conformation of the protein which results in a lower electrophoretic mobility. Once converted into the mature form, the receptors are transported to the plasma membrane, possibly in small coated vesicles, where they can move into coated pits to begin the cycle of endocytosis.

Structure-function relationship

According to the amino acid sequence of the protein and to the intron-exon organization of the gene, the mature receptor has been divided in five functional domains, which are schematically represented in Fig. 7. Each domain, separately, contributes to the overall function of the receptor, so that the disruption of a single domain does not abolish the functions of the remaining domains. Most of the information available on the structure-

function relationship in the LDL receptor has been obtained by studying the effect of naturally occurring and site-directed mutations in the LDL receptor gene on the receptor protein structure and activity. The recent development of techniques which allow to reproduce *in vitro* naturally occurring mutations or to create new ones and the possibility to have them expressed in a biological system by cell transfection, has greatly contributed to the present knowledge of the function of the various domains.

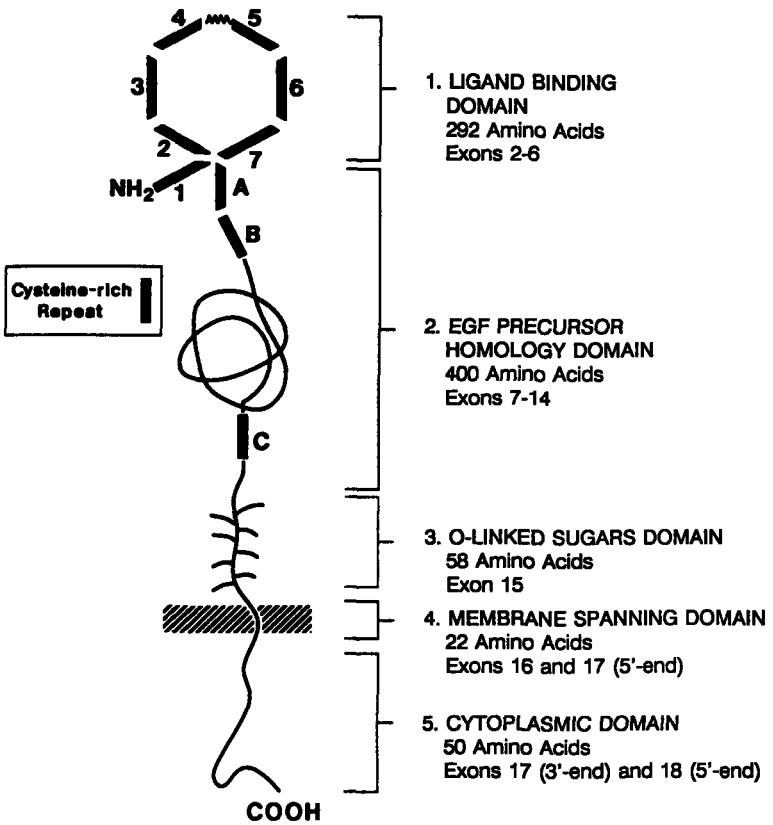

Figure 7. Schematic illustration of the LDL receptor protein and its organization in domains (from Esser et al., Journal of Biological Chemistry, 1988, 263: 13282-13290, reprinted with permission).

The first domain consists of a 292 amino acid sequence at the *N*-terminal of the mature protein and forms the ligand-binding domain. It consists of seven repeats, each comprising about 40 amino acids, six of which are cysteines. All six cysteine residues are involved in the formation of disulphide bonds (Lehrman et al., 1987b) either between or within repeats.

As a result, this domain exists in a tightly folded state, which probably preserves the receptor activity during its exposure to the acidic endosomal pH (Goldstein et al., 1985). Each of the seven repeats shows homology to a single 40-residue sequence in human complement C9, and is therefore designated as complement repeat. Each repeat also contains a cluster of negatively charged amino acids located at the *C*-terminal, which appears to be partially complementary to the positively charged receptor-recognition sequences in apoE and apoB. This suggests that the negatively charged clusters represent the multiple binding sites for apolipoprotein ligands (Innerarity et al., 1984; Knott et al., 1985).

Analysis of the effects produced by naturally occurring and site-directed mutations indicates that the individual repeats display different functions. Hobbs et al. (1986) described a FH patient with a deletion of the sixth repeat, resulting in a truncated receptor which was able to bind β-VLDL with normal affinity, but not LDL. The protein was recognized by the monoclonal antibody IgG-C7. Transfection of receptor-negative Chinese hamster ovary (CHO) cells with various constructs of the LDL receptor gene (Van Driel et al., 1987) demonstrated that repeat 1, which contains the epitope for the monoclonal antibody IgG-C7, is not involved in ligand binding. Similar experiments performed by Esser and Russell (1988a) and Esser et al. (1988b) led to the conclusion that repeat 5, and possibly also 4, represent an absolute requirement for binding of both LDL and β-VLDL. Repeats 2 plus 3 and 6 plus 7 are required for maximal binding of LDL, but not of β-VLDL. Apparently, the binding of LDL requires much more stringent conditions than that of β-VLDL, possibly due to the fact that, in the binding of LDL, only one molecule of apoB100 interacts with one binding site, while the binding of β-VLDL is mediated by several apoE molecules (Myant, 1990).

The second domain consists of a stretch of approximately 400 amino acid residues which display a strong degree of sequence homology to the epidermal growth factor (EGF) precursor (Yamamoto et al., 1984; Südhof et al., 1985). This domain contains three imperfect cysteine-rich repeats of about 40 amino acids each, designated A, B and C. Although they contain 6 cysteine residues as do the repeats in domain 1, the two classes of repeats differ from each other in the spacing so denouncing their different evolutionary origin.

There are two potential *N*-glycosylation sites in domain 2. Because of structural reasons, their presence is highly likely. The role of the *N*-linked sugars on the properties and activity of the receptor is not clear yet. Studies performed by Filipovic (1989) revealed that the *N*-linked oligosaccharides are not needed for intracellular translocation and expression at the cell surface. However, the inhibition of *N*-glycosylation by tunicamycin results in a reduction in the number of receptors at the cell surface by about 50%, possibly due to an increased premature degradation of the unglycosylated protein.

Mutational analysis revealed some of the functional properties of this domain. Selective deletion of the entire domain does not affect the transport and processing of the receptor, nor the ability to bind β-VLDL (Davis et al., 1987a). However, repeats A and C are required for binding of LDL on the cell surface (Esser et al., 1988b). Specific sequences within this domain, in particular repeats A and B, are a prerequisite for the release of bound ligand from

the receptor at acid pH, which is an essential event for receptor recycling (Davis et al., 1987b).

The third domain is a sequence of 58 amino acids, located just outside the plasma membrane. It is highly enriched in serine and threonine residues, to which the *O*-linked oligosaccharides are attached. No specific function could be assigned to this domain, based on deletion analysis. Receptors lacking the entire clusters of *O*-linked sugars behave similarly to the normal receptors and are transported to the cell surface, where they bind and internalize LDL, and recycle to the plasma membrane at normal rates (Davis et al., 1986a). In addition to the clustered *O*-linked sugars, the LDL receptor also contains a few scattered *O*-linked sugar chains, whose location is as yet unknown (Davis et al., 1986a). Studies on a mutant line of CHO cells (Kingsley et al., 1986), with a markedly defective synthesis of *N*- and *O*-linked sugars, revealed that the LDL receptors synthesized by these cells are rapidly degraded, without being processed. This suggested that the isolated and not the clustered *O*-linked sugars are essential for the stability of the receptor.

The fourth domain is a stretch of 22 hydrophobic amino acids spanning the membrane and anchoring the protein with the right orientation: *C*-terminal on the cytoplasmic face and *N*-terminal on the opposite face. No other specific function could be attributed to this domain, as indicated by the low interspecies homology found (Yamamoto et al., 1986). Mutational analysis showed that, in most cases, receptors lacking the transmembrane region are directly secreted into the medium; in a few cases, the receptors reach the cell surface, but, since they are loosely incorporated into the membrane, they are eventually excreted (Lehrman et al., 1987c). Therefore, in both situations, the absence of this domain results in the inability of the receptor to bind LDL.

The fifth domain is a *C*-terminal sequence of 50 amino acids, located on the cytoplasmic side of the plasma membrane (Russell et al., 1984). The analysis of naturally occurring and artificial mutations has revealed that the major function of this domain is to target the LDL receptor to coated pits in the plasma membrane (Lehrman et al., 1985; Davis et al., 1986b). In particular, the first 22 amino acids of this sequence (residues 790-811) appear to be required for rapid internalization of the cell surface LDL receptor (Chen et al., 1990). This sequence must include an aromatic amino acid (tyrosine, phenylalanine or tryptophan) at residue 807; replacement of the wild type Tyr^{807} by a non-aromatic residue markedly diminishes internalization (Davis et al., 1986b, 1987a). In the human LDL receptor, this position is part of a tetrameric sequence, asparagine, proline, valine and tyrosine (NPVY). A similar sequence, NPXY, where X stands for any amino acid, is conserved in LDL receptors from six different species and in two members of the LDL receptor gene family (LDL receptor-related protein and rat GP330). A NPXY sequence has also been found in the cytoplasmic domain of at least 10 other cell surface receptors, some of which have been located in coated pits. These considerations and extensive mutational analysis confirmed the role of the NPXY sequence in directing cell surface receptors to coated pits (Chen et al., 1990).

1.6 The LDL receptor gene

The human LDL receptor gene spans approximately 45 kb of DNA on the short arm of chromosome 19 (Francke et al., 1984; Lindgren et al., 1985).The full-length messenger RNA (mRNA) is 5.3 kb long, including a 2.5 kb 3'-untranslated region. Three copies of the *Alu*-repeats have been found in this region (Yamamoto et al., 1984; Hobbs et al., 1985). *Alu*-repeats are also present in several introns of the gene. Although the function of the *Alu* sequences in the human genome is not known, it has been suggested that they might be partially responsible for the high frequency of deletion and insertion mutations in the human LDL receptor gene (Myant, 1990).

The structure of the LDL receptor gene

The gene is divided into 18 exons and 17 introns (see Fig. 7) (Südhof et al., 1985). There is a strong correlation between structural domains in the protein and exon distribution in the LDL receptor gene. Exon 1 encodes 21 hydrophobic amino acids that comprise the signal sequence. Exons 2-6 encode the ligand-binding domain, which consists of the seven cysteine-rich repeats. Exons 2, 3, 5 and 6 each encode a single repeat, whereas exon 4 encodes three repeats. It has been suggested by Südhof et al. (1985) that the seven repeats arose by successive duplication of an ancestral exon and that introns originally present between repeats III-IV and IV-V have been lost during evolution. In line with the duplication hypothesis, another remarkable aspect of the structure of the exons encoding the seven repeats is that all introns of this region interrupt codons at the identical position, namely after the first base of the codon. Hence, each exon of the binding domain can be spliced out in the mRNA, without altering the downstream reading frame.

Exons 7-14 encode the 400 amino acid sequence, which shares homology with the human EGF precursor. Like the repeats in the binding domain, each one of the three cysteine repeat units (A, B and C) of the third domain are encoded by a single exon.

Exon 15 encodes 58 amino acids, rich in serine and threonine, which are the site of attachment of the *O*-linked sugars.

Exon 16 and the 5'-end of exon 17 encode 22 hydrophobic amino acids which constitute the membrane spanning region.

The 3'-end of exon 17 and the 5'-end of exon 18 encode the 50 amino acids forming the cytoplasmic domain. The remaining part (2.5 kb) of exon 18 represents the 3'-untranslated region.

Comparison of the deduced amino acid sequence of the LDL receptor from different species (human, bovine, rabbit, rat, mouse and *Xenopus laevis*) (Russell et al., 1983; Yamamoto et al., 1984, 1986; Lee et al., 1989; Hoffer et al., 1993; Mehta et al., 1991a,b) showed that the protein is highly conserved. The most conserved region is the cytoplasmic domain with more than an 86% identity among species. The least conserved regions are the signal peptide and the *O*-linked sugar domain, both of which are invariably rich in

glycosylated serine and threonine. Studies performed in the toad *Xenopus laevis* (Mehta et al., 1991a,b) showed that the LDL receptor expressed in this species contains all the functional domains and regulatory sequences expressed in the human receptor, thus indicating that all the functional elements of the LDL receptor were assembled in the present form more than 350 million years ago.

Regulation of the expression of the LDL receptor gene

We have discussed earlier that the modulation of the LDL receptor expression is mediated primarily by an alteration of the receptor synthesis, which is reflected by parallel changes in the concentration of LDL receptor mRNA and, primarily, by changes in the rate of transcription of the gene. The 5'-flanking promoter region of the LDL receptor gene contains specific sequences which are responsible for the regulation of transcription (Goldstein and Brown, 1990; Südhof et al., 1987a). These are located within 200 bp of the translation initiator methionine codon and consist of three imperfect repeats of 16 bp each, two TATA-like sequences and a cluster of mRNA transcription initiation sites. The role of these regulatory sequences has been extensively investigated. By using mutated hybrid promoters, Südhof et al. (1987b) demonstrated that all three repeats and the 5' TATA-like sequence are required for normal transcription. Each of the three repeats includes a 10 bp sequence homologous to the GC-rich consensus sequence which serves as a recognition site for the general transcription-activator protein termed Sp1 (Kadonaga et al., 1986). DNAse footprinting has shown that purified Sp1 binds *in vitro* to repeats 1 and 3, but not to repeat 2 (Dawson et al., 1988). Repeat 2 contains an eight nucleotide, positive sterol regulatory element, designated SRE-1. Mutational analysis of this sequence showed that SRE-1 prevents the induction of transcription in the presence of sterols. Since the coordinate action of all three repeats is required for transcription of the gene, the following model has been proposed: repeats 1 and 3 are activated by Sp1; SRE-1 acts as an enhancer that synergizes with the Sp1 sites to promote transcription only in the absence of sterols (Smith et al., 1990; Briggs et al., 1993; Wang et al., 1993).

Besides cellular sterols, other factors such as cellular growth and hormones can modulate LDL receptor transcription. Growth activation of fibroblasts by platelet-derived growth factor (PDGF) and lymphocytes by mitogens (phytohemagglutinin) results in an increased LDL receptor expression (Mazzone et al., 1990). This transient induction of expression occurs in the presence of protein synthesis inhibitors. This is resistant to suppression by sterols and is independent of new protein synthesis (Leitersdorf et al., 1989b; Mazzone et al., 1989; Cuthbert et al., 1989). Second messengers, such as 8-bromo-cAMP, are also involved in the regulation of LDL receptor transcription (Auwerx et al., 1989), although their mode of action has not been clarified yet.

An additional aspect to be considered, with regard to the regulation of the gene expression, is the failure of the normal counter allele to compensate for the defective one in case of heterozygous FH. This was already apparent in the early studies of Goldstein et al. (1976). When the LDL receptor activity of normal and heterozygous FH fibroblasts (which

express only half of the functional receptors) were compared in the presence of increasing amounts of cholesterol in the medium Goldstein and Brown (1976) found that, at any cholesterol concentration used, the number of receptors expressed by the heterozygous cells was always half that expressed by the normal cells. This suggested that each allele responds independently to some signal, other than the total activity of the receptors present on the cell surface.

1.7 Mutations at the LDL receptor gene locus in relation to FH

Shortly after defects in the LDL receptor protein activity had been associated with FH (Brown and Goldstein, 1974a, 1976), studies on the biosynthesis and function of the LDL receptor protein in fibroblasts derived from FH patients revealed the heterogeneity of the disease (Tolleshaug et al., 1982, 1983). Since then, LDL receptor mutations have been classified according to their phenotypic effect on the protein function. The isolation and purification of the LDL receptor protein (Schneider et al., 1982) and, subsequently, the cloning of the LDL receptor gene and complete characterization of the full-length cDNA (Yamamoto et al., 1984; Südhof et al., 1985) represented a breakthrough towards the molecular characterization of mutations in the LDL receptor gene of FH patients. In the following five years, 42 different mutations were characterized genetically and biochemically (for review, see Hobbs et al., 1990). In the last two years, the exponential improvement of molecular genetic techniques, which allow the detection of single nucleotide changes, resulted in the identification and characterization of an impressive additional number of FH-causing mutations. The most updated list (Hobbs et al., 1992) includes 150 mutations found mainly in homozygous FH patients from 14 different countries. A wide spectrum of different mutations is represented among those reported including insertions, deletions, nonsense, missense, frameshift, splicing, scattered along the entire gene, thus confirming the genetic heterogeneity of the disease. Some 105 out of 150 are point mutations or small deletions/insertions (< 25 bp) and these are summarized in Fig. 8. The remaining 45 represent large deletions and insertions, which have been mapped by Southern blotting or molecular cloning and these are summarized in Fig. 9.

Traditionally, as mentioned above, mutations have been and still are classified according to the phenotyping effect on the protein. It is worthy mentioning that this distinction is somewhat arbitrary since many LDL receptor alleles produce proteins that fall into more than one class.

Classification of mutations

Class 1 mutations fail to produce immunoprecipitable LDL receptor protein (null allele). They represent 17% (37 out of 150) of the total number of mutations: the most frequent types of mutation are nonsense and frame-shift mutations randomly distributed among the

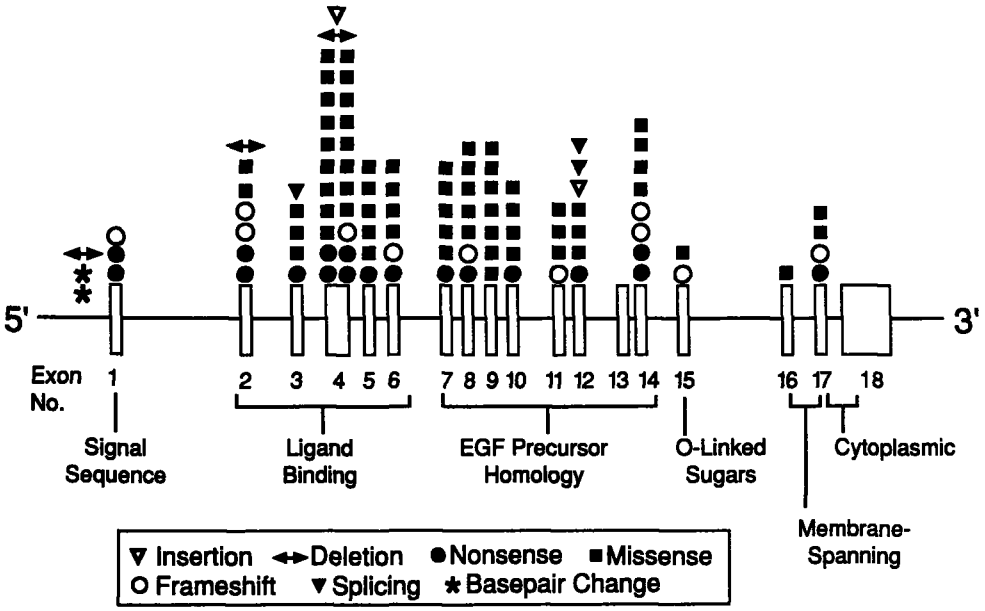

Figure 8. Point mutations and small in-frame deletions/insertions (< 25 bp) in the LDL receptor gene in individuals with FH. Exons are shown as vertical boxes and introns as the lines connecting them. The map is drawn to approximate scale (from Hobbs et al., Human Mutation, 1992, 1: 445-466, reprinted with permission).

exons. These mutations often produce mRNA that is normal in size but reduced in concentration (Hobbs et al., 1988). Hobbs et al. (1992) suggest that the absence of receptor protein in fibroblasts from FH patients carrying this type of mutation may be due either to a rapid turnover of the mRNA or to accelerated degradation of the receptor protein. Three Class 1 alleles contain deletions that include the promoter region. These alleles produce no mRNA or protein. Six Class 1 alleles produce mRNA of abnormal size, two caused by splicing mutations and four by large deletions (Hobbs et al., 1992).

Class 2 alleles produce receptor proteins that fail to reach the cell surface, due to an abolished (Class 2A) or defective (Class 2B) transport between the endoplasmic reticulum, where the protein is synthesized, and the Golgi apparatus, where the post-translational processing of the receptor protein takes place. Class 2 mutations at the LDL receptor locus are very common; they cluster in the exons which encode two domains: two-thirds have been found in the ligand-binding domain and one-third in the EGF precursor homology region. Nearly all (8 out of 9) Class 2A mutations are located in this domain. The presence of several missense mutations in this region, which is the most highly conserved in the receptor protein (Mehta et al., 1991a,b), indicated that single amino acid substitution easily disrupt its structure (Hobbs et al., 1992). Class 2B are missense mutations, in-frame deletions and insertions most of which have been found to be clustered in repeat 5 of the ligand-binding

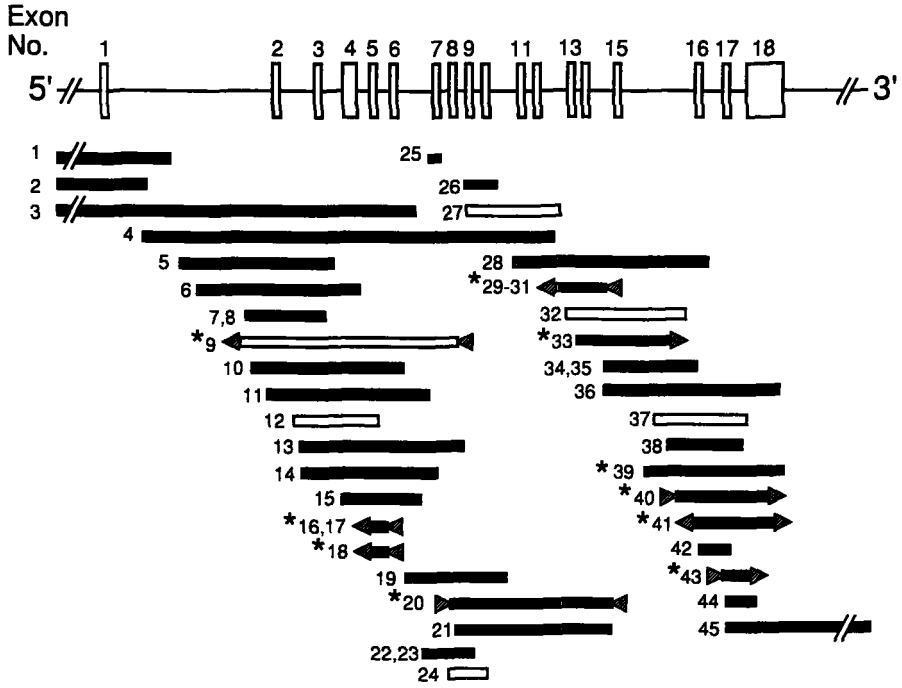

Figure 9. Large deletions and insertions in the LDL receptor gene in individuals with FH. Exons, shown as vertical boxes, are separated by introns, which are drawn to approximate scale. The extent of each deletion (solid bar) or insertion (open bar) is shown below the gene map. Rearrangements involving *Alu* repeats are denoted by stippled arrowheads that indicate the orientation of the repeat element. An asterisk (*) denotes those mutations where the deletion or insertion joint has been sequenced (from Hobbs et al., Human Mutation, 1992, 1: 445-466, reprinted with permission).

domain. This is the only one, among the seven ligand-binding repeats, which is required for the binding of both apoB and apoE. Mutations in this repeat, presumably disrupting the formation of disulphide bonds, prevent the correct folding of the protein which results in an impaired intracellular transport. This type of mutation is also found in the Watanabe Heritable Hyperlipidemic Rabbit (WHHL), the animal model for FH (Yamamoto et al., 1986).

Class 3 mutations encode proteins that are synthesized and transported to the cell surface, but fail to bind LDL (binding-defective alleles). Some of them retain the ability to bind β-VLDL. As anticipated by the phenotype expressed, most of the mutations in this class involve in-frame deletions or insertions in the ligand-binding domain, disrupting the cysteine-

rich repeats which constitute this domain. As discussed previously with regard to the structure-function relationship of the LDL receptor protein, the retained ability of binding β-VLDL depends on whether or not the mutation involves repeat 5. A few Class 3 mutations have been found in the EGF precursor homology region. These are large rearrangements affecting repeats A and C, which are required for the binding of LDL.

Class 4 (internalization-defective) mutations encode receptors that bind LDL on the cell surface but are unable to cluster in coated pits and thus do not internalize LDL. Seven mutant alleles of this kind have been identified and subdivided into two groups: mutations that alter the cytoplasmic domain only (Class 4A), and mutations that involve the cytoplasmic domain and the adjacent membrane spanning region (Class 4B). The most revealing of the Class 4A mutation is the J.D. mutation (Davis et al., 1986b), in which a cysteine substitutes a tyrosine at amino acid 807. Mutational analysis at this locus led to the identification of the sequence requirement for targeting the receptor protein to the coated pits (Davis et al., 1987a; Chen et al., 1990). Class 4B mutations are large deletions producing truncated receptors which lack both the membrane-spanning and the cytoplasmic domain. In most cases, these receptor molecules are secreted from the cell, so that binding at the cell surface is almost completely abolished, and only a minor fraction of receptors, which remain adherent to the cell membrane, display some binding activity (Aalto-Setälä, 1988; Lehrman et al., 1987c).

Class 5 (recycling-defective) mutations specify receptors that, after binding and internalization, do not release the ligand in the endosome, thus interrupting the round trip of endocytosis. Class 5 mutations prevalently occur in the EGF precursor homology region. The occurrence of recycling-defective mutations (Miyake et al., 1989; Van der Westhuyzen et al., 1991) in this domain led to the finding that growth factor repeats A and B mediated the acid-dependent release of the ligand from the receptor, an essential step in receptor recycling (Davis et al., 1987b).

Molecular diagnosis of FH

The most impressive features of rearrangements and point mutations in the LDL receptor gene is that they are widely heterogeneous and, except for those mutations with a higher frequency in a small population due to a founder effect, they appear to be unique. These considerations together with the large size of the LDL receptor gene, and its organization in exons separated by large introns, have significantly retarded the development of a simple strategy for the identification of mutations on a routine basis. To optimize the efficacy of the therapeutic intervention in heterozygous FH, an early diagnosis of the disease is required. An appropriate pharmacological treatment together with a suitable diet and life-style can delay the onset of CAD and therefore dramatically improve the life expectations of the patient.

In case of heterozygous FH, an unequivocal diagnosis cannot always be made, especially in children, in whom the clinical signs of the disease are not yet apparent, or in adults who did not develop xanthomas or severe CAD. In these cases, the presence of hypercholesterolemia or CAD in other members of the family and its segregation with a particular

haplotype (Leitersdorf et al., 1989c) helps to give a diagnosis.

A rapid laboratory test, capable of detecting the molecular defect responsible for the disease, would provide a much more accurate and reliable tool for the individual diagnosis of FH. Indeed, strenuous efforts have been made in the past decade to apply DNA analysis for diagnostic purposes. In this respect, two complementary approaches have been used: (*i*) development of techniques which enable to identify and characterize the largest possible number of mutations causing the disease, such as single strand conformation polymorphism (SSCP) (Hobbs et al., 1992; Loux et al., 1993) or denaturing gradient gel electrophoresis (DGGE) (Top et al., 1992, 1993) and (*ii*) development of rapid, specifically designed techniques to be applied in the screening of large population samples for known mutations (Defesche et al., 1992; Gudnason et al., 1993; Koivisto et al., 1993; Talmud et al., 1991; Top et al., 1990). The combination of these two strategies, together with the exponential improvement of the DNA techniques available, is expected to produce, in a near future, a DNA assay to be routinely used in the diagnosis of FH and, ultimately, a comprehensive overview of all the existing mutations.

1.8 Outline of this thesis

The studies presented in this thesis investigate different genetic and biochemical aspects of the LDL receptor function and its involvement in cellular cholesterol metabolism. The uptake of LDL by several experimental murine tumours was studied in Chapter 2. *In vivo* and *in vitro* studies were performed to verify that rapidly proliferating tissues, requiring cholesterol for membrane synthesis, actively take up LDL by increasing the expression of LDL receptors.

In Chapter 3 we show that the LDL receptor protein in Epstein Barr virus transformed lymphocytes (EBV-L) shares the same properties as the fibroblast LDL receptor. Therefore, we suggest that this cell line may offer an alternative to fibroblasts in the study of LDL receptor protein abnormalities in FH patients.

In both HepG2 cells and freshly isolated human hepatocytes, the LDL receptors are much less responsive to down-regulation by extracellular LDL than the LDL receptors in fibroblasts. To explain the marked difference between HepG2 cells and fibroblasts in their ability to modulate LDL receptor activity in response to extracellular cholesterol, we studied the intracellular processing of LDL in both cell types (Chapter 4). VLDL and lipoprotein lipase treated VLDL (LPL-VLDL), representing VLDL remnants, are taken up by hepatic LDL receptors as is the LDL itself. For these particles, apoE rather than apoB mediates the binding to the receptors. In Chapter 5 the intracellular processing of LPL-VLDL and VLDL as compared to LDL was studied, in order to evaluate the effect of apoE mediated binding of these particles on their intracellular pathway and, consequently, on the overall cellular cholesterol homeostasis.

It is known that lipolysis of chylomicrons and VLDL by LPL improves the binding of both these particles to their hepatic receptors. However, it has also been reported that LPL itself enhances the binding of apoE containing particles to the LRP. Chapter 6 describes the effect of LPL on the binding and intracellular processing of VLDL and LDL in cultured HepG2 cells and in fibroblasts.

In Chapters 7, 8 and 9 we describe different approaches used for studying and characterizing mutations in the LDL receptor gene, causing FH.

In Chapter 7 we studied a LDL receptor mutation in a homozygous FH patient. The characterization of the underlying molecular defect and the effect on the protein structure and biological activity are described.

A PCR-based strategy, allele-specific transcript PCR, was applied, in Chapter 8, to determine the prevalence of allele-specific mRNA deficiency of the LDL receptor gene in a group of heterozygous FH patients. This study revealed that about 25% of heterozygous FH patients carry a mRNA deficient LDL receptor allele.

In Chapter 9, a group of 32 heterozygous FH patients was screened by denaturing gradient gel electrophoresis (DGGE) for the presence of point mutations or small deletions/insertions in the LDL receptor gene. We show that some of the sequence variations detected by DGGE represent known exon-bound polymorphisms and we present evidence indicating that the remaining sequence variations identified are most likely to be FH-causing mutations.

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Chapter 2

ASSIMILATION OF LOW DENSITY LIPOPROTEINS BY EXPERIMENTAL TUMOURS IN MICE

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Biochim. Biophys. Acta 1989; 1003: 301-306 (reprinted with permission).

Summary

We have studied the uptake of ^{125}I-labelled low-density lipoprotein (LDL) by seven experimental murine tumours *in vivo*. Four tumours (Lewis Lung carcinoma, B-16, MS-2 and Colon 26) showed a higher relative uptake of lipoprotein as compared to the liver, two (L-1210 and P-388) had a very low lipoprotein uptake, while lipoprotein uptake by tumour M5 was similar to that of the liver. The data was confirmed by tracing tissue uptake of lipoproteins using [^{14}C]sucrose-labelled LDL. These *in vivo* findings correlated well with the *in vitro* specific binding of ^{125}I-β-VLDL to membranes prepared from tumours, thus suggesting that the expression of the LDL receptor in the tumours is related to the *in vivo* uptake of lipoprotein. Further analysis of the LDL receptor by ligand blotting showed that the tumour receptor has several of the liver LDL receptor characteristics (including apparent Mr, sensitivity to proteinases, and Ca^{2+} requirement for lipoprotein binding). In short, our data show that experimental murine tumours express the LDL receptor and suggest that the relative high *in vivo* uptake of LDL is determined by the elevated LDL-receptor expression in the tumours.

Introduction

Replicating cells require cholesterol for membrane synthesis (1). Cholesterol is acquired either from endogenous synthesis or, preferably, by assimilation of LDL from plasma (2,3).

LDL binds to specific receptors on cellular membranes, initiating a process that eventually leads to the internalization and degradation of the lipoprotein by the cell (4). This pathway is effective in several tissues (5) and accounts for about 80% of the lipoprotein catabolism, the liver being the most effective site of LDL removal from the circulation (5). Recently, several reports have appeared describing how in tumours cells cultured *in vitro*, the pathway of LDL assimilation via the receptor is very active (6-12). Furthermore, membranes prepared from tumour cells of mice showed a several-fold increase of LDL binding over other tissues, including liver (13). These data suggest that some tumour cells may depend also *in vivo* upon LDL for their optimal growth and this finding may represent a clue to a better targeting of cytotoxic drugs at tumours (14,15).

We have shown that LDL are taken up with high efficiency by a murine tumour cell line (MS-2) *in vivo* (8). The purpose of this work is to verify the possibility that other experimental tumours *in vivo* also actively assimilate LDL, and to compare the characteristics of the receptor protein expressed by the tumour to those of the LDL receptor present in the liver.

Materials and Methods

All animal used were obtained from Charles River (Calco, Italy) and had free access to water and food throughout the study.

The following tumour cell lines were used:

- L-1210: ascitic leukaemia induced with methylcholantrene (16). The tumour cells (10^5) were injected intraperitoneally in CDF1 male mice, 8-10 weeks old, under sterile conditions in 0.2 ml of Hank's medium.The experiments were performed 9 days after inoculation of the tumour cells.
- P-388: ascitic leukaemia (17). Cells (10^5) were injected in BDF1 male mice as previously for the L-1210 line. The animals were used 16 days after tumour inoculation.
- M-5: a spontaneous ovary carcinoma (18). 50-80 mg of tumour "tissue" were injected in the upper limb in C57 BL female mice. The experiments were performed 3 weeks after the inoculation.
- LLC: the tumour is a spontaneous lung carcinoma (19). Cells (10^6) were injected intramuscularly in the right hind leg of C57 BL mice. The experiments were performed 9-10 days after the inoculation.
- Colon 26: a colon adenocarcinoma (20). The tumour tissue (50-80 mg) was injected in the hind leg of BALB/C female mice 8-10 weeks old. The experiments were performed 25 days after the tumour inoculation.
- B-16: a spontaneous melanoma (21). Tumour cells (10^6) were injected in the scapular region of C57 BL/6 male mice. Experiments were performed 15 days after the inoculation.
- MS-2: a tumour line derived from the moloney virus-induced sarcoma (22). Cells (10^6) were injected intramuscularly in the right hind leg of female BALB/C mice. The experiments were performed 12-14 days after the inoculation.

Lipoproteins

Human LDL was separated from the plasma of normolipidemic donors by ultracentrifugation for 24 h at 12°C in a 60 Ti Beckman rotor (40,000 rpm) in the density range 1.020-1.050 g/ml (22). LDL was labelled with ^{125}I to a specific activity of 120-250 cpm/ng of LDL protein as described (23). More than 95% of the LDL protein was apoB and more than 99% of the radioactivity was precipitable with 10% trichloroacetic acid (TCA). Less than 10% of the LDL-associated radioactivity was chloroform-extractable. LDL was stored at 4°C under sterile conditions and used within 5 days after the isolation. β-VLDL was separated by ultracentrifugation from the plasma of cholesterol-fed rats (d, 1.006 g/ml) and labelled with ^{125}I to a specific activity of 70-170 cpm/ng (23). Less than 1% of the radioactivity was TCA non-precipitable and less than 9% was lipid-associated.

LDL was injected i.v. during the morning in control and tumour-bearing animals. Animals were killed 24 h after the LDL injection and samples of the following tissues were

obtained: liver, abdominal muscle, brain, abdominal adipose tissue, spleen, intestine and tumour. The tissues were washed three times in 5 ml of Krebs-Ringer medium at 25°C in Petri dishes on a rotating platform. Tissue samples were then blotted dry, weighed and counted for radioactivity in a Packard counter. Data were calculated as cpm in 1 g of tissue/cpm in 100 μl of plasma. In some experiments, [^{14}C]sucrose-labelled lipoproteins were used to confirm these data. LDL were labelled using [^{14}C]sucrose (Amersham, U.K.) essentially as described by Pittman et al. (24). The specific activity was 18 μCi/mg protein. Lipoproteins were injected i.v. during the morning. The animals were killed 24 h later. Tissues were removed and washed as described for ^{125}I-labelled lipoproteins. Tissues were digested, processed and counted for radioactivity in a β-counter (Packard, U.S.A.), using an appropriate standard curve to correct for quenching.

Membrane preparation and binding studies

The expression of the LDL receptor in tumours and liver was evaluated by studying the binding of rat β-VLDL to membranes prepared from these tissues. Membranes were prepared as described by Kovanen et al. (25) and stored at -80°C for up to 4 months without apparent loss of binding activity. Binding was performed as described (26) at pH 8 and 0°C for 1 h. Bound lipoprotein was separated from the unbound by ultracentrifugation using a Beckman 42.2 LP rotor (30 min at 40,000 rpm, and 4°C). The pellet was washed once with fetal calf serum, reprecipitated and counted for radioactivity. The nonspecific binding comprised the ^{125}I-lipoprotein bound in the presence of a 50-fold excess of unlabelled lipoprotein.

Ligand blotting studies

To further confirm the results obtained in the binding studies we performed ligand-blotting experiments. These experiments give further information on the nature of the receptor protein since the apparent M_r can be detected. The membranes were solubilized with 40 mM octyl β-glucoside and the soluble protein was separated by 6% polyacrylamide gel electrophoresis in the presence of 1% SDS. The separated proteins were electrophoretically transferred onto a nitrocellulose sheet and the nitrocellulose replica was incubated, after saturation of the unbound sites with bovine serum albumin (3% in PBS, pH 7.4), with ^{125}I-β-VLDL in 0.15 M NaCl/0.09 M Tris (pH 7.4). After three washes with the same buffer, the nitrocellulose was air-dried and processed for autoradiography. The intensity of the radioactive band was determined by scanning of the X-ray films, the M_r was calculated from the mobility relative to standards and to bovine adrenal LDL receptor (27). Sensitivity of the binding to proteolytic enzymes was assessed by immunoblotting after incubation at 37°C for 15 min of the membranes with pronase (10 μg/ml of membrane protein). Ca^{2+} requirement for LDL binding was determined by addition of 10 mM Na_2-EDTA to the incubation mixture. β-mercaptoethanol was also added (3% final concentration) to assess the effect of disulphide-bond cleavage on the lipoprotein binding ability of the receptor.

Results

Lipoprotein uptake by the tissues

Preliminary experiments were performed to address the question of whether 24 h was the appropriate time to evaluate the uptake of LDL by the tissues. In these experiments the tissue radioactivity (cpm/g of tissue/100 μl of plasma) was similar at 6, 12 and 24 h (data not shown). For further experiments we chose the 24 h time. Table 1 shows the relative uptake for the various tissues in control and tumour-bearing animals; the data are expressed in relative terms as well as absolute values for the liver. Taking the liver as 100% no difference was detected in the relative LDL uptake by other tissues between control and tumour-bearing animals, suggesting that the tumour does not affect to any significant extent the relative uptake of LDL by other tissues.

Four out of seven experimental tumours studied were more active than the liver in assimilating lipoproteins on a weight basis. The two ascitic tumours had a very low uptake of lipoproteins (Table 1). The M5 tumour had a relatively low uptake of LDL (about 60%) as compared to the liver; however, if the core of the tumour was separated from the fibrous cap that surrounded the tumour itself, the uptake of lipoprotein by the core was much higher (140%), thus suggesting that the fibrous cap does not contribute effectively to the uptake of lipoproteins. Additional experiments using [^{14}C]sucrose-labelled lipoproteins also showed that the tumours (LLC and MS-2) took up more lipoproteins than the liver (Table 2). A low uptake tumour (P-388), on the other hand, did not take up or internalize lipoprotein very efficiently (Table 2). These data add further strength to the observation made using ^{125}I-LDL, since 24 h lysosomal accumulation of lipoprotein labelled with sucrose, a marker of lipoprotein uptake by the tissues, showed the same trend.

To address the question of whether the lipoprotein uptake by the tumours depends on the day on which the experiment was performed, we carried out a time-course study with the following tumours: L-1210 (low-LDL uptake tumour), B-16 and LLC (high-LDL-uptake tumours). The data are shown in Table 3. No significant differences in the lipoprotein uptake were detected at various days after the tumour inoculation. This observation demonstrated that the data reported in Table 1 do not depend upon the day on which the experiment was performed. The LLC tumour showed a trend towards lower uptake activities with time; these differences, however, were not statistically significant.

Binding of lipoproteins to liver and tumour membranes

The results of the binding of rat β-VLDL to liver and tumour membranes are reported in Table 4. All the tumours tested except for the L-1210 and P-388 showed a high specific binding of β-VLDL. Saturation curves, performed with the low-uptake tumours P-388 and L-1210 and high-uptake tumours LLC and MS-2, demonstrated that the specific binding curve is due to a single, saturable binding site with a similar K_d for the liver and the tumour (Table 5), thus suggesting an increased receptor density in the tumour membrane preparation.

Table 1. Relative uptake of different tissues of mice after i.v. injection of ^{125}I-LDL.
Mice were injected i.v. with ^{125}I-LDL and killed 24 h later. Tissues were removed, washed and counted as described under Materials and Methods. Data were calculated as cpm/g tissue and this value corrected for plasma radioactivity (100 μl of plasma). The mean value of liver uptake in each experimental group was taken as 100%. Actual values for liver were 3.7 ± 0.7 (L-1210), 4.1 ± 0.9 (P-388), 3.9 ± 0.7 (LLC), 3.5 ± 0.8 (B-16), 4.3 ± 0.7 (MS-2), 4.2 ± 0.9 (M5), 3.6 ± 0.9 (Colon 26), 4.3 ± 0.8 (controls).

	Liver	Spleen	Muscle	Intestine	Adipose tissue	Brain	Tumour
L-1210 (6)[a]	100 ± 19.1	73.8 ± 8.2	18.0 ± 8.2	46.9 ± 8.9	42.6 ± 21.3	16.4 ± 8.7	10.9 ± 4.3
P-388 (6)	100 ± 21.2	99.8 ± 27.2	19.7 ± 9.3	39.5 ± 10.2	29.6 ± 14.1	5.2 ± 6.3	3.2 ± 2.8
LLC (12)	100 ± 18.3	79.8 ± 21.2	17.8 ± 6.3	48.3 ± 11.1	38.6 ± 12.2	14.2 ± 5.2	190.2 ± 39.2
B-16 (8)	100 ± 22.2	78.9 ± 19.4	15.5 ± 6.2	38.9 ± 14.7	39.5 ± 13.9	16.3 ± 6.2	205.4 ± 57.2
MS-2 (8)	100 ± 15.5	77.4 ± 12.7	16.9 ± 6.1	53.9 ± 6.4	48.6 ± 26.1	14.1 ± 4.1	154.2 ± 40.1
M5 (8)	100 ± 22.4	92.7 ± 27.6	16.7 ± 5.6	50.7 ± 11.9	49.7 ± 18.2	11.3 ± 4.6	58.8 ± 19.6
Colon 26 (8)	100 ± 26.2	103.8 ± 28.7	24.6 ± 7.9	53.5 ± 12.8	47.8 ± 19.2	12.6 ± 6.2	192.2 ± 39
Controls (24)	100 ± 18.6	88.1 ± 16.9	18.7 ± 6.1	46.3 ± 12.9	46.7 ± 22.8	14.8 ± 8.1	-

[a] Values in parentheses are the number of animals used in each experiment.
[b] All data are significantly different from liver ($P = 0.01$), MS-2 was $P < 0.05$. Duncan's Test. In each experiment the liver mean value was taken as 100%.

Table 2. Uptake of ^{14}C-labelled LDL by the liver and tumours in mice.
Animals (control and tumour-bearing mice) were injected i.v. with ^{14}C-labelled lipoproteins. The animals were killed 24 h later and the tissues removed, washed, weighed and processed for counting of the radioactivity as described under Materials and Methods. All values are dpm/g tissue. (Mean ± S.D., n = 6).

	Liver	Tumour
Controls	27680 ± 3424	-
LLC	33447 ± 3958	61710 ± 8612[a]
MS-2	34839 ± 4729	68220 ± 7408[a]
P-388	28612 ± 5782	2412 ± 1927

[a] $P < 0.01$ vs. liver.

Table 3. Time-dependency of tumour/liver ratio on uptake of ^{125}I-LDL.
Tumour-bearing mice were injected i.v. with ^{125}I-LDL; 24 h later, the animals were killed and the livers and tumours were processed for counting of radioactivity as described under Materials and Methods.

	Tumour/liver ratio					
Day from the tumour injection:	7 (5)[a]	8 (6)	9 (5)	10 (6)		
L-1210	0.10 ± 0.03	0.13 ± 0.06	0.13 ± 0.04	0.12 ± 0.07		
Day from the tumour injection:	13 (5)	14 (5)	15 (6)	16 (5)	17 (5)	18 (5)
B-16	1.76 ± 0.32	1.60 ± 0.22	1.5 ± 0.31	1.82 ± 0.27	2.12 ± 0.41	1.90 ± 0.36
Day from the tumour injection:	8 (5)	10 (5)	12 (5)	16 (4)		
LLC	1.71 ± 0.44	1.42 ± 0.43	1.48 ± 0.32	1.37 ± 0.31		

[a] Values in brackets represent the animals used in each study. The ratio is for data calculated as cpm/g tissue.

Table 4. Specific binding of β-VLDL to liver and tumour membranes.
Data are expressed as ng of β-VLDL protein bound per mg of membrane protein. Assays were performed as described under Materials and Methods at a final concentration of 0.5 μg/ml of lipoprotein. Specific binding was the binding in presence of a 50-fold excess of unlabelled lipoproteins. (Mean ± S.D., n = 4).

	Tumour	Liver
Controls	-	50 ± 8
L-1210	60 ± 12	61 ± 11
P-388	38 ± 3	59 ± 6
LLC	212 ± 27[a]	42 ± 8
MS-2	136 ± 31[a]	64 ± 13
M-5	60 ± 4	40 ± 9
B-16	128 ± 11[a]	50 ± 6
Colon 26	126 ± 13[a]	48 ± 11

[a] $P < 0.01$ vs. liver.

Table 5. Apparent K_d and B_{max} for binding of ^{125}I-LDL β-VLDL to liver and tumour membranes. Data reported are from two separate experiments performed using different membrane preparations. Every determination was performed in duplicate and the data did not differ by more than 12%. Data are relative to specific binding and were calculated according to Scatchard.

	K_d (μg/ml)	B_{max} (ng β-VLDL protein/ mg liver or tumour membranes)
Liver	1.0, 1.5	145, 129
L-1210	0.9, 0.7	89, 69
P-388	1.5, 1.2	67, 82
LLC	0.4, 0.6	461, 367
MS-2	1.3, 1.6	238, 270

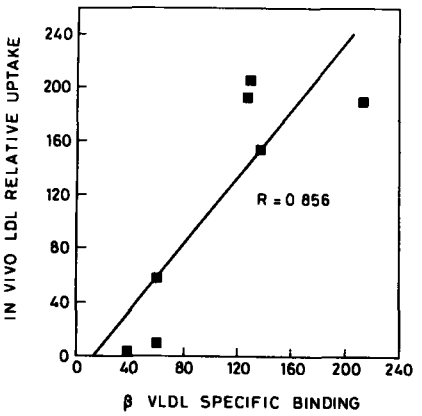

Figure 1. Correlation between *in vivo* uptake of ^{125}I-LDL and *in vitro* binding of ^{125}I-β-VLDL by the seven tumours studied. The correlation coefficient was 0.856 ($P < 0.05$). For experimental details, see Materials and Methods.

In addition, the LLC tumour showed an increased affinity that was not detected in any other tumour tested. In separate experiments we also studied the binding of ^{125}I-LDL to liver and tumour membranes. The LDL binding increased 3-fold in the Colon 26 and approximately 4-fold in the LLC tumour (data not shown). These findings are in agreement with the data obtained with ^{125}I-β-VLDL (Table 4).

Figure 1 shows the correlation between the specific binding data and the *in vivo* assimilation of LDL. A significant positive correlation was found ($r = 0.856$, $P < 0.05$)), suggesting that *in vitro* data can predict the *in vivo* uptake of LDL by the tumour. A positive correlation was also found with total binding of β-VLDL ($r = 0.847$, $P < 0.05$) owing to the fact that nonspecific binding of β-VLDL was low (10-18 ng/mg membrane protein) and bore no correlation at all with the uptake of LDL. These data were further stressed by the results of the ligand blotting experiments. We observed a M_r of approximately 120 kDa for the LDL receptor both in liver and tumours with no appreciable differences in M_r between the two tissues (Fig. 2). This figure reports the data for the LLC membranes (high uptake and binding) and for the L-1210 (low uptake and binding). The data is consistent with a high

expression of LDL receptor in the LLC tumour and a low expression in the L-1210 tumour, in agreement with the *in vitro* binding and *in vivo* kinetic data. Similar results were also obtained for the other tumours (data not shown). Furthermore, the sensitivity to proteolytic treatment and the Ca^{2+} requirement for LDL binding is also maintained in these receptors (Fig. 3). Altogether, these findings suggest that the LDL receptor expressed by the experimental tumours studied is very similar, if not identical to the LDL receptor expressed by the liver in mice.

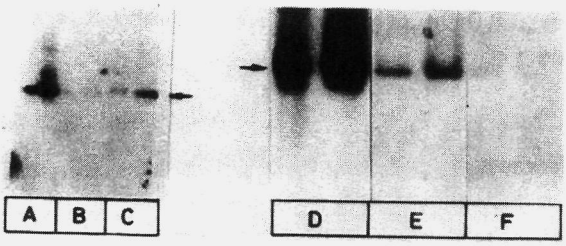

Figure 2. Autoradiograms of the ligand blotting analysis of LDL receptor using membranes from bovine adrenals, mice liver and tumours. 10 and 20 μg of membrane protein was applied to the gels. Lane A, bovine adrenals; B, liver from LLC tumour-bearing animals; C, LLC tumour; D, bovine adrenals; E, livers from L-1210 tumour-bearing animals; F, L-1210 tumour. The apparent M_r of the liver and tumour receptor are comparable to that of bovine adrenals. The arrow indicates the position of the 116 kDa standard.

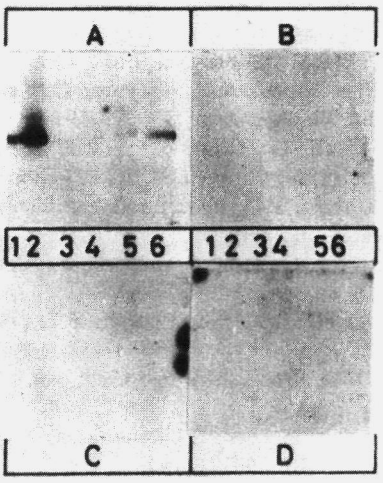

Figure 3. Effect of EDTA, pronase and β-mercaptoethanol on the LDL binding to the receptor. 10 and 20 μg of membrane protein were applied to the gels. Panel A: LDL binding to adrenal (lane 1, 2), liver (lane 3, 4), and tumour (lane 5, 6) membranes. Panel B: effect of pronase on LDL binding. Lanes are numbered as in Panel A. Panel C: effect of EDTA on LDL binding. Lanes are as in Panel A. Panel D: effect of β-mercaptoethanol on the binding of LDL. Lanes are as in Panel A.

Discussion

Many tumours *in vitro* take up and catabolize lipoproteins for their cholesterol requirements (6-12). We have previously shown that a high uptake of LDL is also present *in vivo* using the MS-2 tumour in mice (8). A similar finding was also reported by Hinds et al. using a soft tumour (7). The purpose of our work was to further extend our observation to other tumours *in vivo*, with the goal of evaluating whether a high uptake of LDL is a hallmark of experimental tumours *in vivo*. A second purpose of this study was to verify whether the LDL receptor expressed by the tumour *in vivo* is comparable to the

hepatic receptor. The *in vivo* data (Table 1) show that the uptake of LDL is high in all but two of the experimental tumours tested. The uptake (relative to the liver) was the highest in the B-16 tumour and the lowest in the L-1210 and P-388 tumours. We have previously shown that both the receptor-mediated and the receptor-independent pathways contribute to the uptake of LDL by the tumour (8). In the present study, we did not address this question directly; the positive correlation between *in vivo* and *in vitro* receptor-binding data, however, suggests that *in vivo* the receptor pathway plays an important role (see Fig. 1).

It has been suggested that human LDLs are degraded by nonspecific pathways (5) in rodents (28). Modification of arginine or lysine of LDL, however, dramatically reduces the catabolism of human LDL in rats (29), and ^{125}I-labelled human LDLs are rapidly removed in rats whose liver LDL receptors were induced by 17α-ethynylestradiol treatment (30) and bind efficiently to the solubilized receptor in ligand blotting experiments (31). Furthermore, Pittman et al. (32) have shown that the tissue accumulation of [^{14}C]sucrose-labelled human LDL is very similar to that of [^{14}C]sucrose-labelled rat LDL with the possible exception of the spleen. We feel, therefore, that human LDL can effectively trace LDL receptors in rodents although we cannot exclude a lower sensitivity using this approach as compared to the use of homologous lipoproteins.

A second point to discuss is that the data obtained with ^{125}I-labelled lipoproteins reflect the uptake of LDL by the tissue for a few hours before the animals were killed. LDLs, in fact, once internalized, are degraded rapidly by the tissues (1). In preliminary experiments we have shown that at 6 and 12 h after ^{125}I-labelled LDL inoculation, the liver/tumour radioactivity ratio was similar, suggesting that the time elapsed between inoculation of ^{125}I-LDL and killing of the animals does not affect the data i.e. a steady-state is reached. The possibility exists, however, that LDL may be taken up or degraded at different rates by the tumour cells as compared to other organs. This would affect the results obtained, since a low rate of degradation would result in an overestimation of the uptake of LDL and vice versa a high rate in an underestimation. To address this question, we have performed experiments with [^{14}C]sucrose-labelled LDL (24) in tumour-bearing animals. After internalization of the lipoprotein, sucrose is trapped in the lysosomes, thus providing a good estimate of the relative role of a given tissue in the LDL catabolism. The data are in agreement with those obtained by using ^{125}I-LDL (Table 2). We therefore concluded that several experimental tumours take up LDL at a high rate.

The rate of tumour growth may vary, and as a consequence, could affect the relative uptake of lipoproteins, thus making crucial the reproducibility of results at the time after inoculation of the tumour that the values were measured. This question was addressed performing experiments at different days of tumour growth. The data suggest that, in the time interval we have tested, this variable does not play a significant role. Wether this pathway is present at an early stage of tumour growth still remains to be determined.

We then addressed the question of whether the receptor in the tumours presents the same characteristics as the hepatic LDL receptor. The ligand blotting data show that the

receptor protein in the tumours is similar, if not identical, to the counterpart in the liver (Fig. 2). Invariably, receptor proteins with the same M_r were detected in the liver and tumours. If these findings are considered together with the binding data, in which no major changes of K_d were detected but only increases of B_{max} (Table 4), and experiments in which pronase sensitivity, Ca^{2+}-dependency for binding, and the requirements for disulphide bonding of cysteine were demonstrated (Fig. 3), it is reasonable to conclude that the same receptor protein is expressed in the tumours and liver of the animals we studied. The LLC tumour represents a possible exception with a lower K_d compared to the liver membranes. Whether this finding represents an artefact of the binding study or a true difference, however, remains to be addressed. The fact that the LDL receptor in the tumours shares all the properties of the hepatic LDL receptor, including molecular mass, suggests that the variation by the apparent K_d might be related to the experimental conditions used; alternatively, changes in the membrane composition might have affected the LDL receptor affinity for the ligand.

In conclusion, our data *in vivo* extend previous observations on the expression of LDL receptors by experimental tumours suggesting that in several, but not all, tumours this pathway is very active. Furthermore, the *in vitro* experiments indicate that a high expression of LDL receptors was found in tumours with high LDL assimilation *in vivo* (Table 4, Fig. 1). This correlation suggests that *in vitro* data may predict the *in vivo* findings; *in vitro* screening would therefore allow us to differentiate tumours with high affinity for LDL *in vivo* for possible delivery of drugs via this pathway (14,15).

Acknowledgements

This work was supported, in part, by a grant from CNR Progetto Finalizzato Oncologia to A.L.C. The secretarial help of Miss Marta Colombo is acknowledged.

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Chapter 3

CHARACTERIZATION OF THE LOW DENSITY LIPOPROTEIN RECEPTOR IN EPSTEIN-BARR VIRUS TRANSFORMED LYMPHOCYTES

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Biochim. Biophys. Acta 1990; 1044: 127-132 (reprinted with permission).

Summary

Non-dividing human lymphocytes were transformed upon infection with the Epstein-Barr virus (EBV) into lymphoblasts which are capable of continuous growth in culture. We studied the properties of the LDL receptor in EBV-transformed human lymphocytes (EBV-L) by binding experiments and by ligand blotting.

EBV-L show a high affinity binding of LDL in the same order of magnitude as found with fibroblasts; EBV-L obtained from a homozygous familial hypercholesterolemic (FH) patient fail to express LDL receptor activity. Similar to that of fibroblasts, the LDL receptor activity in EBV-L is Ca^{++}-dependent and is down-regulated by the presence of an exogenous source of cholesterol in the medium. The LDL receptor protein of EBV-L has an apparent molecular weight of 130,000.

Since our results show that EBV-L display a LDL receptor protein similar to the LDL receptor present in fibroblasts, we conclude that in comparison with other cell types EBV-L offer a suitable model system to investigate LDL receptor protein abnormalities in FH patients.

Introduction

Cells require cholesterol for membrane synthesis. In most cell types cholesterol is acquired either from endogenous synthesis or, preferably, from circulating LDL through a receptor-mediated pathway. This pathway involves high affinity surface binding, internalization by endocytosis and lysosomal degradation of LDL (1).

Familial hypercholesterolemia (FH) is an inherited disorder in which the uptake of LDL by the LDL receptor is impaired, leading to an accumulation of LDL in the plasma and consequently to premature coronary artery disease (2).

FH originates from mutations within the LDL receptor gene, resulting in a defective receptor synthesis, or processing, or in an inability of the receptor to bind or internalize LDL (3-9). Restriction fragment length polymorphism (RFLP) analyses used in these studies, revealed that mutations in the LDL receptor gene are widely heterogeneous. Furthermore, by RFLP analysis only large rearrangements in the gene can be detected. Point mutations or small deletions/insertions representing more than 90% of all LDL receptor mutations (9) are difficult to detect by simple DNA techniques, unless more laborious methods, such as DNA sequence analyses, are applied. Therefore screening for LDL receptor mutations in a large population sample needs complementary approaches. We suggest that additional (small) mutations could be revealed by studying the LDL receptor polymorphism on the protein level by isoelectric focusing and/or by two-dimensional electrophoresis. Furthermore, mutations affecting the rate of synthesis or the rate of conversion of the LDL receptor protein from the precursor into the mature form could be characterized by pulse-chase experiments followed

by immunoprecipitation, SDS polyacrylamide gel electrophoresis and autoradiography (10).

The study of the LDL receptor protein abnormalities requires an established cell line from each subject under investigation. Therefore a suitable cell line should fulfil the following conditions: (*i*) primary cultures should be easily isolated; (*ii*) the cell lines, once established, should be maintained in culture for long periods of time or stored without loss of viability; and (*iii*) the cell line should be able easily to provide large amounts of LDL receptor protein.

At present, cultured skin fibroblasts represent the most used cell line for the characterization of LDL receptor mutants. However, because of the number of advantages Epstein-Barr virus transformed lymphocytes (EBV-L) have over fibroblasts (continuous cell culture for a long period of time without senescence, short doubling time, growth in suspension), we wondered whether EBV-L could offer an alternative to fibroblasts to study LDL receptor protein abnormalities in FH patients. Moreover, lymphocytes are readily available and easily transformed upon infection with the EB virus (11).

The aim of the present study was to characterize the properties of the LDL receptor activity in EBV-L. By LDL binding studies and by ligand blotting experiments we found that the LDL receptor in EBV-L shows the same characteristics as demonstrated in fibroblasts (12,13).

Materials and Methods

Transformation of lymphocytes with the Epstein-Barr virus

10 mls of heparinized blood were diluted with the same volume of Balanced Salt Solution (BSS), layered on 15 ml Ficoll Isopaque and centrifuged for 30 minutes at 400 x g at room temperature. White blood cells were then collected and washed at room temperature with 3 volumes of BSS by centrifugation for 10 minutes at 100 x g. If residual red blood cells were present, cells were resuspended in lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4) and incubated for 2 minutes to lyse erythrocytes and then diluted at least 10 times with BSS. Lymphocytes were separated from lysing buffer by centrifugation, washed twice in 10 ml of BSS and counted. The final pellet of white blood cells (approximately 1 x 10^7 cells) was incubated for 1 hour at 37°C with 6 ml of the Epstein-Barr virus solution, derived from the B95-8 Marmoset lymphoblastoid cell line (14). Cells were then harvested by centrifugation for 5 minutes at 100 x g, resuspended in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS) and 1% phytohemagglutinin (PHA, reagent grade, Wellcome) and plated in Multiwell tissue culture clusters (Costar) at a density of about 1 x 10^6 cells/ml.

Cells were maintained at 37°C in 5% CO_2 atmosphere and refed with the medium containing PHA until they began to proliferate (after 2-6 weeks). EBV-L were then transferred to tissue culture flasks (50 ml, Costar) and maintained in RPMI 1640 medium, supplemented with 15% FCS.

Cell culture

Human skin fibroblasts were cultured in 24 wells dishes in Dulbecco's modification of Eagle's medium (DMEM) supplemented with penicillin (100 i.u./ml), streptomycin (0.1 mg/ml) and 10% (v/v) heat inactivated FCS.

EBV-transformed lymphocytes were grown in 650 ml flasks (Costar) in RPMI 1640 medium containing penicillin and streptomycin and supplemented with 15% FCS at a density of approximately 1 x 10^6 cells/ml.

Lipoproteins

LDL was isolated from serum of normolipidemic donors by density gradient ultracentrifugation by the method of Redgrave et al. (15). A narrow density fraction (density 1.04-1.05 g/ml) was used. LDL was immediately labelled with ^{125}I (Amersham) to a specific activity of 80-120 cpm/ng, exactly as described in (16). After labelling the LDL sample was dialysed and stabilized as described previously (17). The stabilized ^{125}I-labelled LDL was stored at 4°C and used within two weeks. Less than 1% of the radioactivity was soluble in 10% (v/v) trichloroacetic acid (TCA). When not labelled with ^{125}I, LDL was immediately stabilized with 1% HSA and extensively dialysed against subsequently Phosphate Buffered Saline (PBS) and DMEM or RPMI 1640 supplemented with penicillin and streptomycin.

Lipoprotein-depleted serum (LPDS) was obtained by ultracentrifugation of serum at a density of 1.21 g/ml followed by extensive dialysis against subsequently PBS and DMEM or RPMI 1640 supplemented with penicillin and streptomycin.

β-VLDL was separated by ultracentrifugation from the plasma of cholesterol-fed rabbits ($d < 1.006$ g/ml) and labelled with ^{125}I (16) to a specific activity of 100-200 cpm/ng. After labelling, β-VLDL was extensively dialysed against PBS. More than 99% of the radioactivity was precipitable with 10% TCA. ^{125}I-β-VLDL was stored at 4°C and used within one week.

Binding studies

Twenty four hours prior to the experiments, cells were washed twice with PBS and then incubated with 10% LPDS medium, or 10% LPDS medium containing different amounts of human LDL, as indicated in the figure legends. Washings of EBV-L were carried out by harvesting and centrifugation for 5 minutes at 800 x g. Immediately before the binding experiment, cells were washed again with the respective medium containing 1% HSA instead of 10% LPDS. Thereafter the EBV-L were transferred to 1.5 ml Eppendorf tubes. To the 2 cm^2 wells (fibroblasts) or Eppendorf tubes (EBV-L), the indicated amounts of ^{125}I-LDL were added in the presence or absence of 300 μg of unlabelled LDL/ml.

After 3 hours of incubation at 37°C the medium was removed for determination of LDL degradation as described previously (17). After removal of the incubation medium, cells were cooled to 0°C and washed exactly as described by Goldstein et al. (18).

When total-cell association of ^{125}I-LDL was measured, the washed cells were dissolved in 0.2 M NaOH and an aliquot of the cell lysate was counted. Another aliquot of the cell lysate was used for the protein determination by the method of Lowry et al. (19) with bovine

serum albumin (BSA) as standard.

When ^{125}I-LDL binding and internalization were measured separately, the washed cells were incubated with 0.05% trypsin in buffer 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 and 0.02% EDTA at 37°C (20). The radioactivity released into the trypsin buffer reflects the amount of ^{125}I-LDL bound to the cell membrane. The radioactivity remaining cell-associated represents the amount of ^{125}I-LDL internalized.

When the binding experiment was carried out at 4°C, the total amount of cell-associated ^{125}I-LDL represents the amount of ^{125}I-LDL bound to the plasma membrane. The amount of ^{125}I-LDL that was degraded, bound or cell-associated after incubation in the absence of unlabelled LDL, subtracted by the amount of ^{125}I-LDL that became degraded, bound, or cell associated in the presence of excess of unlabelled LDL, represents the "receptor-mediated" or "high-affinity" degradation, binding or cell-association.

Ligand blotting

Fibroblasts were seeded into 75 cm^2 flasks (Costar) and maintained as described above. EBV-transformed lymphocytes obtained from the normal and the FH subject were maintained as described above in 650 ml flasks (Costar) at a density of approximately 1 x 10^6 cells/ml.

After 24 hours of pre-incubation with medium containing 10% LPDS, cell extracts were prepared according to the method of Van Driel (21). Briefly, cells from different flasks were combined, washed in buffer A (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM PMSF, 0.5 mM leupeptin, 10 mM EDTA, 10 mM EGTA, 10 mM N-ethyl maleimide, 2.2% DMSO) and solubilized in lysis buffer (buffer A plus 1% Triton X-100).

The soluble protein extracts were clarified by centrifugation at 100.000 x g for 1 hour and frozen immediately. The protein concentration of the extracts was assayed by the BCA protein assay reagent (Pierce) for non-ionic detergent containing samples.

Cell extracts were subjected to SDS polyacrylamide gel electrophoresis and ligand blotting was performed as described by Daniel (22). The dried blots were autoradiographed for 2-5 days using pre-flashed films and intensifying screens.

Results

Figure 1(a) shows the receptor-mediated binding, internalization and degradation of ^{125}I-LDL by EBV-transformed lymphocytes from a healthy subject. Saturation is achieved at low LDL concentration (approximately 10 μg/ml), indicating that a high affinity binding site is present. High affinity binding, internalization and degradation of ^{125}I-LDL are not expressed in EBV-L derived from a homozygous FH patient (Fig. 1b). Furthermore, the high affinity uptake and degradation of ^{125}I-LDL by EBV-L is totally inhibited by the presence of 3 mM EDTA in the incubation medium (data not shown), indicating that the LDL receptor activity in EBV-L is Ca^{++}-dependent, as previously reported in fibroblasts (23).

In Fig. 2 the binding of ^{125}I-LDL to EBV-L is compared with that of fibroblasts. The binding curves showed that for both cell lines the binding of ^{125}I-LDL reached saturation at 10 to 15 ng of ^{125}I-LDL added. In addition, the two cell lines showed similar amounts of maximum ^{125}I-LDL binding.

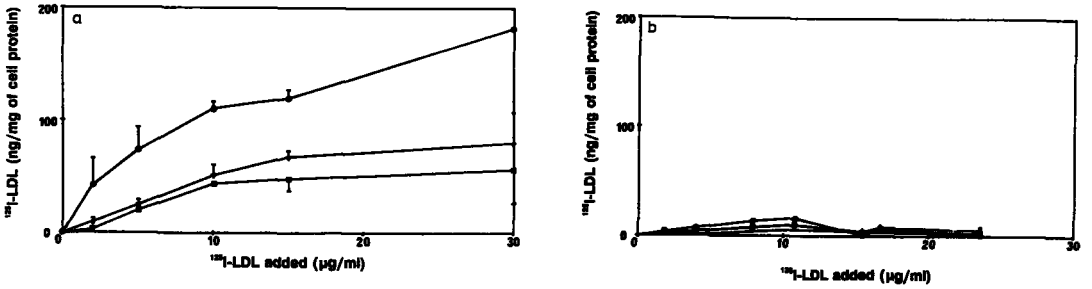

Figure 1. Receptor-mediated binding, internalization and degradation of human ^{125}I-LDL by EBV-lymphoblasts from a normal subject (a) and from a homozygous FH subject (b). Cells were pre-incubated in medium supplemented with 10% LPDS for 24 hours. Binding (■), internalization (▼) and degradation (●) were measured after incubation at 37°C with ^{125}I-LDL as described in the Materials and Methods section. Results are the mean of triplicate measurements ± S.D.

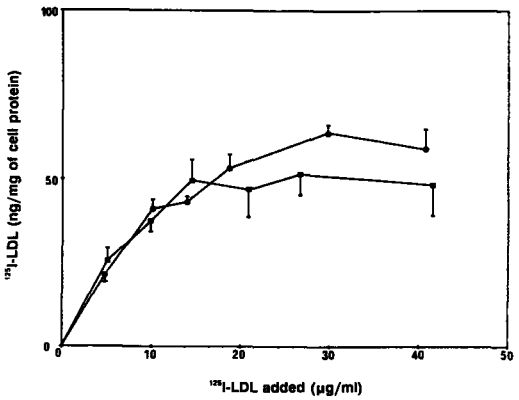

Figure 2. Saturation curves of the high affinity binding of human ^{125}I-LDL to EBV-transformed lymphocytes (●) and fibroblasts (■). Cells were pre-incubated in medium supplemented with 10% LPDS for 24 hours. Binding was measured after 3 hours of incubation with ^{125}I-LDL at 4°C as described in Materials and Methods. Results are the mean of triplicate assays ± S.D.

Studies on human skin fibroblasts have shown that the expression of the LDL receptor activity is regulated by the presence of cholesterol in the medium (23,24). In order to compare EBV-L with fibroblasts in this respect, we measured the receptor-mediated degradation of LDL after preincubation of the cells for 24 hours in medium supplied with increasing concentrations of LDL. As shown in Fig. 3, the receptor activity declined with increasing concentrations of LDL during the preincubation period. Maximum suppression occurred at 50 μg/ml of LDL with fibroblasts (80% inhibition) and at 80-100 μg/ml of LDL with EBV-L (approximately 50% inhibition).

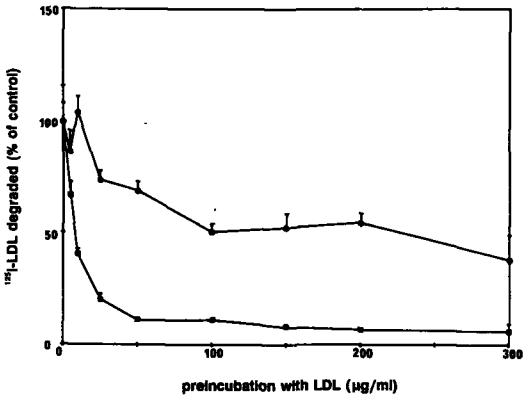

Figure 3. Effect of preincubation of fibroblasts (▪) and EBV-L (●) with increasing concentrations of LDL on LDL receptor activity. Cells were incubated for 24 hours in medium containing 10% LPDS and different concentrations of LDL for 24 hours as indicated. After washing of the cells, high-affinity degradation was measured after 3 hours of incubation at 37°C with 10 µg/ml of ^{125}I-LDL as described in Materials and Methods. Results are expressed as percentage of control and they represent the mean of triplicate assays ± S.D.

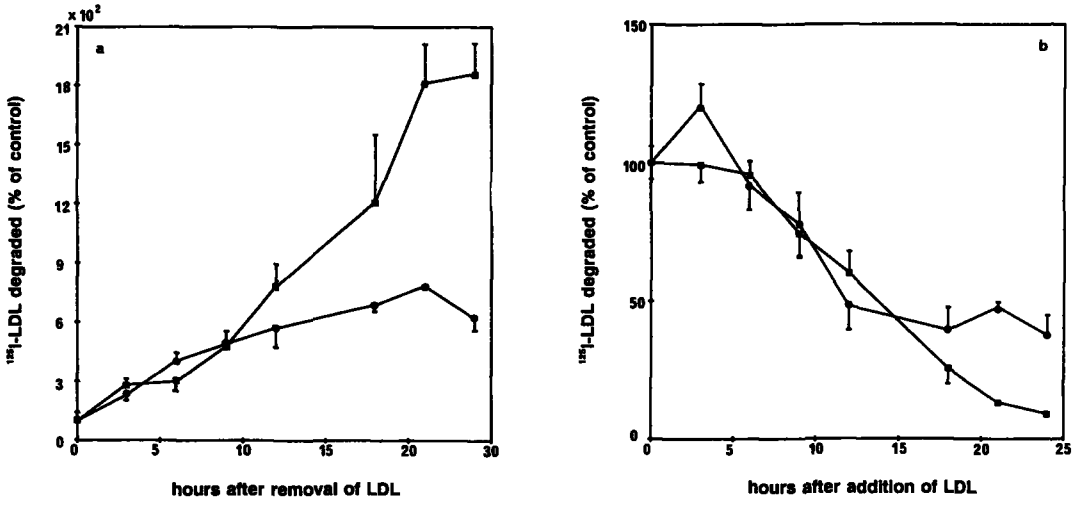

Figure 4. Time-course of the up- and down- regulation of the LDL receptor activity in fibroblasts and EBV-L upon depletion and addition of LDL, respectively. For measuring upregulation (a), fibroblasts (▪) and EBV-L (●) were incubated for 24 hours in medium containing 10% LPDS and 300 µg/ml of human LDL. Thereafter, the cells were washed and incubated for the indicated time periods in the same medium but without LDL. For measuring down-regulation (b), fibroblasts (▪) and EBV-L (●) were incubated for 24 hours in medium containing 10% LPDS. Thereafter, the cells were washed and incubated for the indicated time periods with the same medium but supplied with 300 µg/ml of human LDL. High affinity degradation was measured after 3 hours of incubation at 37°C with 10 µg/ml ^{125}I-LDL as described in Materials and Methods. Results are expressed as percentage of control (time 0) and they represent the mean of triplicate assays ± S.D.

Figure 4 shows the time-course of the up- and down-regulation of the LDL receptor activity in fibroblasts and EBV-L upon depletion and addition of LDL, respectively. Both cell lines appeared to be similar in this respect except that both the up- and down-regulation of the LDL receptor in fibroblasts were more pronounced. Time course experiments, performed during a 48 hour period, demonstrated that at 20-24 hours the maximal effect is achieved in both cell lines (data not shown).

To gain further information on the nature of the LDL receptor protein in EBV-L as compared to that of fibroblasts, we performed ligand blotting experiments after SDS polyacrylamide gel electrophoresis of cellular membrane extracts. As shown in Fig. 5a, in extracts of EBV-L, we detected a LDL receptor protein with an apparent molecular weight of approximately 130,000 (lanes 3-4), identical to that detected in human fibroblasts (lanes 1-2). No bands were visible in the extracts of EBV-L obtained from a homozygous FH patient (lanes 5-6). The presence of 10 mM EDTA during the incubation partially inhibited the binding of ^{125}I-rabbit β-VLDL (ligand) to the receptor protein (Fig. 5b). When the ligand blotting was performed in the presence of an excess of unlabelled β-VLDL, no bands were detectable (Fig. 5c).

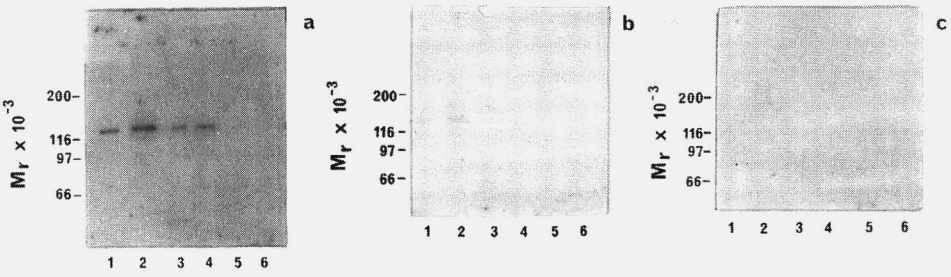

Figure 5. Identification of the LDL receptor in fibroblasts, normal EBV-L and EBV-L obtained from a homozygous FH patient by ligand blotting. Fibroblasts and EBV-L were preincubated for 24 hours in medium containing 10% LPDS; cell extracts were subjected to SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes as described in Materials and Methods. The nitrocellulose blots were incubated with: A) rabbit ^{125}I-β-VLDL (4 μg/ml); B) ^{125}I-β-VLDL plus 10 mM EDTA; C) ^{125}I-β-VLDL plus a 30-fold excess of unlabelled β-VLDL. Lanes 1-2: fibroblasts (50-100 μg of protein, respectively). Lanes 3-4: normal EBV-L (50-100 μg of protein, respectively). Lanes 5-6: EBV-L from a homozygous FH patient (50-100 μg of protein, respectively). The positions of the molecular mass standards are indicated.

Discussion

We presented several lines of evidence that EBV-transformed lymphocytes display LDL receptor activity similar to that of fibroblasts:

(*i*) EBV-L show binding saturation curves in the same order of magnitude than that of fibroblasts; (*ii*) EBV-L obtained from a homozygous FH patient fail to express LDL receptor activity; (*iii*) the LDL receptor in EBV-L is sensitive to EDTA and is regulated by the amount of LDL present in the medium and (*iv*) the LDL receptor protein of EBV-L has an apparent molecular weight of 130,000 similar to that of fibroblasts. We did not directly compare the rate of conversion of the precursor to the mature form, nor the rate of synthesis

of the LDL receptor protein, between both cell lines. However, our results show that in EBV-L the time-course of the up- and down-regulation of the LDL receptor activity, upon changing the amount of LDL in the medium, is almost the same as that found in fibroblasts. This strongly suggests that in both cell lines the turnover rate of the LDL receptor protein is comparable. The fact that the LDL-mediated regulation of the LDL receptor is less pronounced than that found in fibroblasts is most probably due to the relatively high growth rate of EBV-L as compared to fibroblasts.

To study the LDL receptor protein polymorphisms, an established cell line from each subject under investigation is required. A number of cell types could be used for this purpose. In addition to cultured fibroblasts (24,25), LDL receptor activity has been shown to be present in human peripheral blood monocytes (26), freshly isolated human lymphocytes (27), and mitogen-stimulated human lymphocytes (28). For selecting the most useful cell line, a number of requirements should be considered. In particular, primary cultures should be easily isolated; the cell lines, once established, should be maintainable in culture for long periods of time or storable without loss of viability and they should be able to easily provide large amounts of LDL receptor protein.

Data from literature (29) show that phytohemagglutinin activates the proliferation of freshly isolated lymphocytes, transforming them into blast cells. Phytohemagglutinin-stimulated lymphocytes, displaying T cell characteristics, proliferate effectively through a number of cell cycles. It has been suggested that these cells represent a suitable model system for studying abnormalities in the LDL receptor protein (30). However it has been shown that these cells display an optimal proliferation between the second and the fifth day after activation; after that time the rate of proliferation declines rapidly, as the cells enter a lysis phase (31,32).

EBV-transformed lymphocytes, on the contrary, offer all the advantages of an established cell line. The establishment of lymphoid cells is relatively simple and once immortalized by EB virus infection, EBV-L represent an unlimited and abundant source of material (11). Large amounts of LDL receptor protein can be easily obtained by EBV-L as these cells show a high growth rate.

Transformation of human lymphocytes with the Epstein-Barr virus is a common technique for establishing lymphocytes for biochemical studies (33-35). The Epstein-Barr virus is a herpes-like DNA virus that is strongly implicated in the pathogenesis of infectious mononucleosis and is also associated with the Burkitt's lymphoma (36). However, EBV-L can be safely cultured, since these cells carry the latent viral genomes without supporting virus replication. Moreover 70% of the adult population develop antibodies against the Epstein-Barr virus before the age of 40 (37).

Since our results show that the EBV-transformed lymphocytes display a LDL receptor protein similar to the LDL receptor present in fibroblasts, we can conclude that in comparison with other cell types, the EBV-transformed lymphocytes offer a suitable model system for evaluating LDL receptor protein abnormalities in hypercholesterolemic patients.

Acknowledgements

These investigations are supported by The Netherlands Heart Foundation (Project no. 89.057).

Mrs. Clara Horsting-Been and Miss Marisa Horsting are gratefully acknowledged for preparing the manuscript.

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Chapter 4

LOW DENSITY LIPOPROTEINS ARE DEGRADED IN HEPG2 CELLS WITH LOW EFFICIENCY

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Biochem. J. 1993; 290: 509-514 (reprinted with permission).

Summary

In previous studies we have shown that in HepG2 cells, as compared with fibroblasts, the low-density lipoprotein (LDL) receptor is only weakly down-regulated upon incubation of the cells with LDL. To explain this difference in down-regulation of the LDL-receptor activity, we studied simultaneously the intracellular processing of ^{125}I-labelled LDL in both cell lines. Upon incubation of HepG2 cells with ^{125}I-LDL, the appearance of degradation products started at 90 min, whereas in fibroblasts this lag time was only 30 min. The degradation efficiency (representing the ratio degradation/cell association of LDL) in HepG2 was less than 50% of that in fibroblasts up to 5 h of incubation at 37°C. The longer lag time and low efficiency of the degradation of LDL in HepG2 cells were independent of the cell density. Pulse-chase experiments indicated that the internalization rate of surface-bound LDL in HepG2 cells is similar to that of fibroblasts. Endosomal loading of ^{125}I-LDL by incubation at 18°C for 4.5 h, followed by a shift to 37°C, resulted in degradation of LDL within 30 min in fibroblasts, whereas in HepG2 cells the lag time of the degradation was 90 min. In parallel experiments using subcellular fractionation by Percoll-gradient centrifugation of homogenized cells and ^{125}I-tyramine-cellobiose-labelled LDL, we observed that in both cell types LDL is equally rapidly shifted from a low- to a high-density compartment (within 15 min), representing the endosomal and the late-endosomal plus lysosomal compartment respectively. We conclude that in HepG2 cells the cell-bound LDL, upon internalization, goes through the intracellular itinerary at the same rate as in fibroblasts, but that either the fusion between late endosomes and lysosomes or the lysosomal degradation itself is proceeding at a lower efficiency. A low degradation rate of LDL may contribute to explain the relatively weak down-regulation of the LDL-receptor activity in HepG2 cells by LDL.

Introduction

It has been widely demonstrated that the liver is the major site of removal of low-density lipoproteins (LDL) from the circulation, accounting for about 80% of the total LDL catabolism [1]. Up to 70% of LDL is cleared from the plasma via high-affinity binding to receptors gathered in coated pits on the plasma membrane. After binding, the coated pits invaginate, after which endosomes are formed and, upon acidification, the LDL dissociates from the receptor. The receptor returns to the surface, binds another lipoprotein particle and initiates another cycle of endocytosis. After dissociation from the receptor, LDL is delivered to the lysosomes, where the protein component of LDL is hydrolysed to amino acids. The cholesterol released from the degraded LDL leads to a decrease in the cholesterol biosynthesis *de novo*, activates the esterification of cholesteryl esters and suppresses the synthesis of LDL receptors *de novo* by blocking gene expression. These co-ordinated actions

allow the cells to be provided with sufficient cholesterol for metabolic needs without causing over-accumulation of free cholesterol (for review, see [2]).

To study the regulation of LDL-receptor activity and cellular cholesterol homoeostasis, HepG2 cells are often used as a model for human hepatocytes [3-5]. HepG2 cells have been shown to possess functional LDL receptors with properties similar to those of human fibroblasts [3]. Previous studies performed in our laboratory have shown that the LDL receptors in both HepG2 cells and freshly isolated human hepatocytes are much less responsive to down-regulation by extracellular LDL than are LDL receptors in fibroblasts [6,7]. In addition, the LDL-receptor activity in HepG2 cells and human hepatocytes was found to be stimulated 2-3-fold by the presence of cholesterol acceptors such as heavy high-density lipoproteins (HDL), whereas in fibroblasts the LDL-receptor activity was almost insensitive to the presence of heavy HDL.

Studies of the cellular homoeostasis in relation to the LDL-receptor activity in HepG2 cells have suggested that both the LDL cholesterol and the endogenously synthesized cholesterol are primarily directed to a cholesteryl ester pool or, if present, to extracellular cholesterol acceptors, like heavy HDL, rather than to the cholesterol pool involved in the regulation of the LDL-receptor activity [8]. We reasoned that more information about the intracellular pathway of LDL was needed to explain the marked difference between HepG2 cells and fibroblasts in their ability to modulate LDL-receptor activity in response to extracellular LDL-cholesterol. Therefore, we studied the kinetics of binding internalization, distribution into the endosomal-lysosomal compartment and degradation of LDL in both HepG2 cells and fibroblasts. We found that in HepG2 cells the LDL particles are internalized and delivered to the late-endosomal or lysosomal compartment at the same rate as in fibroblasts, whereas in HepG2 cells the degradation of LDL occurs rather inefficiently. This observation may provide an additional explanation for our previous findings that in HepG2 cells the LDL-receptor activity is poorly down-regulated upon incubation of the cells with exogenous (LDL) cholesterol.

Materials and Methods

Materials

Fetal-calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM; cell culture medium) were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Human serum albumin (HSA) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $Na^{125}I$ (sp. radioactivity 13.3 μCi/μg) was purchased from Amersham International (Amersham, Bucks, U.K.). Multiwell cell-culture dishes were from Costar (Cambridge, MA, U.S.A.). Percoll (density 1.13 g/ml) was obtained from Pharmacia (Uppsala, Sweden). Trypsin (from pig pancreas) was from ICN Biomedicals (Cleveland, OH, U.S.A.).

Lipoproteins

LDL was isolated from serum of normolipidaemic donors by density-gradient ultracentrifugation as described by Redgrave et al. [9]. LDL was immediately labelled with ^{125}I as described by Bilheimer et al. [10]. The specific radioactivity ranged from 100 to 150 c.p.m./ng of LDL protein. After iodination, the LDL sample was dialysed against phosphate-buffered saline (PBS, pH 7.4) and stabilized with 1% (w/v) HSA [3]. The stabilized ^{125}I-labelled LDL was stored at 4°C and used within 2 weeks. Less than 1% of the radioactivity was soluble in 10% (w/v) trichloroacetic acid. For ^{125}I-tyramine-cellobiose (TC) labelling, LDL was first dialysed exhaustively against PBS/0.01% (w/v) EDTA. The labelling was then performed exactly as described by Pittman and Taylor [11]. ^{125}I-TC-LDL (specific activity in the range 60-80 c.p.m./ng) was stabilized as above and used within 2 weeks. Whenever unlabelled LDL was used, it was immediately stabilized after isolation with 1% HSA, followed by extensive dialysis against PBS and subsequently DMEM supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

Lipoprotein-depleted serum (LPDS) was obtained by ultracentrifugation of serum at a density of 1.21 g/ml, followed by extensive dialysis of the infranatant against PBS and subsequently DMEM supplemented with penicillin and streptomycin.

Cell culture

HepG2 cells and fibroblasts were cultured in flasks in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 20 mM Hepes, 10 mM $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in equilibration with air/CO_2 (19:1). Then 6-7 days before each experiment, cells were seeded in 2 cm^2 multiwell dishes.

At 24 h before the assays, the cells were washed with Puck's saline [0.136 M NaCl, 5.3 mM KCl, 4 mM $NaHCO_3$, 0.1% (w/v) glucose, 5 mg/l Phenol Red, pH 7.4] and further incubated with DMEM containing 10% (v/v) LPDS instead of FCS.

Binding assays

Each experiment was started by washing the cells three times in DMEM/1% HSA, followed by incubating the cells in the same medium containing 10 μg of ^{125}I-LDL/ml, in the presence or absence of a 50-fold excess of unlabelled lipoprotein. Temperature and duration of the respective incubations are described in the text and in the figure legends. After the incubation with labelled LDL, cells were cooled to 0°C. Degradation of LDL was measured exactly as described in [12]. After removal of the incubation medium, the cells were washed extensively as described in [13]. To measure total cell association, the washed cells were dissolved in 0.2 M NaOH, and a portion of the cell lysate was counted for radioactivity. Another portion of the cell lysate was used for protein determination as described by Lowry et al. [14].

When the internalization of ^{125}I-LDL was measured, cells were washed and further incubated with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in PBS for 10 min at room temperature [15]. The radioactivity released into the buffer represents the amount of ^{125}I-LDL bound to the cell membrane. The radioactivity which remains cell-associated represents the

amount of ^{125}I-LDL internalized.

Values for the specific (receptor-mediated) binding, internalization and degradation were calculated by subtracting the amount of labelled LDL that was bound, internalized or degraded in the presence of a 50-fold excess of unlabelled LDL (non-specific binding) from the amount of labelled LDL that was bound, internalized or degraded in the absence of excess unlabelled LDL (total binding).

Subcellular fractionation using Percoll

HepG2 cells and fibroblasts were subcellularly fractionated on Percoll density gradients as previously described [16]. Cells were seeded in 100 mm dishes (fibroblasts) or 60 mm dishes (HepG2 cells). After incubation for 4.5 h at 18°C in the presence of 10 μg/ml ^{125}I-TC-LDL, cells were washed twice with DMEM/1% HSA to remove the unbound ligand and further incubated at 37°C for the indicated periods of time in medium without ^{125}I-TC-LDL. The cells were then washed extensively with 0.28 M sucrose/2 mM $CaCl_2$/0.01 M Tris/HCl, pH 7.6 (homogenization buffer), and scraped from the dishes with a rubber policeman in the homogenization buffer (1 ml/dish). The cells from three 100 mm dishes (for fibroblasts) or from two 60 mm dishes (for HepG2 cells) were combined for homogenization in a Dounce homogenizer by 20 complete strokes with a tight-fitting pestle. The homogenates were centrifugated at 280 g for 10 min in order to remove remaining intact cells. The supernatants, containing 65-80% of the cell-associated radioactivity, were made 20% in Percoll by addition of 3 ml of 80% (v/v) Percoll in homogenization buffer and adjustment of the total volume of 12 ml with homogenization buffer. After thorough mixing, the samples in Percoll were placed in cellulose nitrate tubes fitting a 50Ti rotor (Beckman) and centrifuged at 10,000 g for 45 min. Fractions (~0.3 ml) were collected by aspiration from top to bottom, and the radioactivity in each sample was counted. The density of each fraction was measured in a PAAR-DMA-45 density meter equipped with a DMA-602M small sample cell (~ 170 μl). The distribution of the lysosomal marker (acid phosphatase) was measured by the method of Torriani [17].

Results

Time course of receptor-mediated association and degradation of ^{125}I-LDL in fibroblasts and HepG2 cells

The receptor-mediated association and degradation (defined as difference between the binding in the absence and in the presence of a 50-fold excess of unlabelled LDL) of ^{125}I-LDL by HepG2 cells and fibroblasts as a function of incubation time at 37°C are shown in Fig. 1(a). In both cell lines, LDL-association increased progressively over the first 2-3 h, before reaching an apparent steady-state level. In fibroblasts, after a lag period of 30 min, the degradation of ^{125}I-LDL proceeds rapidly. However, in HepG2 cells a lag period of 90 min

was observed before the degradation of ^{125}I-LDL started at a slower rate than in fibroblasts. When the degradation efficiency is calculated as the amount of LDL degraded relative to the amount of LDL which becomes cell-associated (Fig. 1b), it is clear that in HepG2 cells the degradation efficiency is less than 50% of that in fibroblasts (at 5 h of incubation). In Fig. 1 10 μg/ml ^{125}I-LDL was used. Similar results were observed in the presence of 20 μg/ml ^{125}I-LDL.

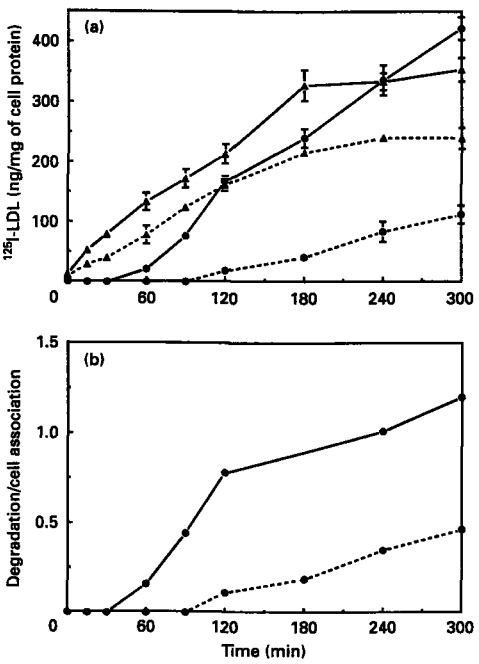

Figure 1. Time course at 37°C of the LDL-receptor-mediated association (▲) and degradation (●) in fibroblasts (———) and HepG2 cells (----). Cells (mean cell density of 61 and 273 μg/well for fibroblasts and HepG2 cells respectively) were incubated with 10 μg of ^{125}I-LDL/ml, with or without a 50-fold excess of unlabelled LDL. Values are means ± S.D. of triplicate incubations. Panel (b) represents the degradation efficiency of LDL calculated from panel (a) as the ratio high-affinity degradation/association in fibroblasts (———) and HepG2 cells (----). This figure shows the results of one representative experiment out of three.

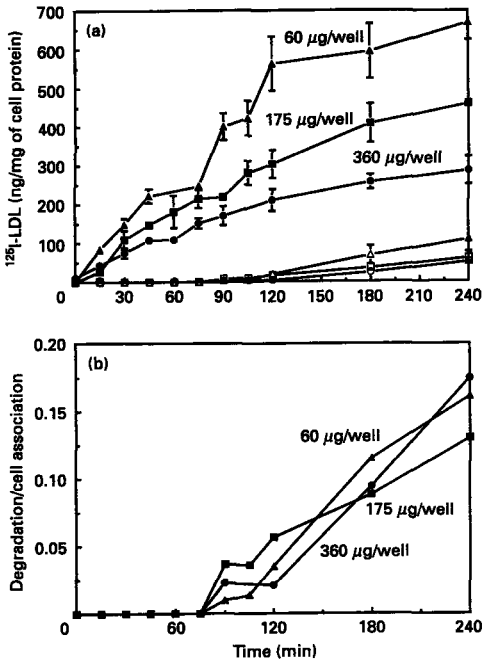

Figure 2. Influence of the cell density on the high-affinity cell association and degradation of ^{125}I-LDL in HepG2 cells. (a) Time course at 37°C of LDL-receptor-mediated cell association (black symbols) and degradation (white symbols) in HepG2 cells seeded at three different cell densities, corresponding to a mean protein content of 360 μg/well (●,○), 175 μg/well (■,□) and 60 μg/well (▲,△). Cells were incubated with 10 μg of ^{125}I-LDL/ml with or without a 50-fold excess of unlabelled LDL. Values are means ± S.D. of triplicate determinations. (b) Degradation efficiency calculated as the ratio degradation/association at each cell density. The symbols are as above. The results are obtained from one representative experiment out of two.

Influence of the cell density on cell association and degradation of ^{125}I-LDL in HepG2 cells

In fibroblast cultures it has been shown that the LDL-receptor activity decreases with an increase in cell density [18]. We wondered whether the same correlation holds true for the expression of the LDL receptor in HepG2 cells and whether this could explain the observed delay and relatively low efficiency of the degradation of LDL in HepG2 cells. As for fibroblasts, with HepG2 cells both the cell association and degradation were higher at lower cell densities, on the basis of cell protein (Fig. 2a). The degradation started after a lag period of about 90 min, irrespective of the cell density. The calculated degradation efficiency appeared thus to be independent of the cell density (Fig. 2b). As a next step, we investigated whether the decreased degradation efficiency of LDL in HepG2 cells could be due to (*i*) a decreased internalization rate of surface-bound LDL, (*ii*) a delayed and less efficient transport of LDL from the early-endosomal to the late-endosomal or lysosomal compartment, or (*iii*) a less efficient degradation of LDL in the lysosomes itself.

Rate of internalization of surface-bound ^{125}I-LDL in fibroblasts and HepG2 cells

The rate of internalization of ^{125}I-LDL was examined by first incubating HepG2 cells and fibroblasts at 4°C with 10 μg/ml ^{125}I-LDL for 2 h, to allow binding of LDL to its cell surface receptors. Thereafter, the cells were washed and further incubated at 37°C. As shown in Fig. 3, most of the cell-bound LDL is already internalized within 5 min, whereas the maximal internalization is reached within 15-20 min with both fibroblasts and HepG2 cells. These data indicate that in HepG2 cells the internalization of surface-bound LDL is similarly rapid as compared with fibroblasts. As a remaining explanation, it is possible that in HepG2 cells either the internalized LDL is not rapidly released from the receptor within the early-endosomal compartment and thus cannot be further processed, or the internalized LDL is normally targeted to the late-endosomal and lysosomal compartment but cannot readily be degraded, due either to an impairment in the late-endosome-lysosome fusion or to a defect in the lysosomal degradation itself.

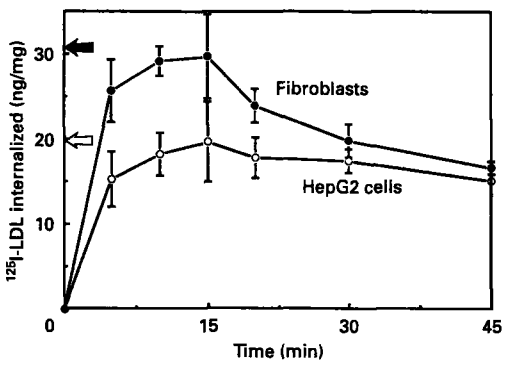

Figure 3. Internalization rate of LDL in fibroblasts (•) and HepG2 cells (○). After 2 h of incubation at 4°C in the presence of 10 μg of ^{125}I-LDL/ml with or without a 50-fold excess of unlabelled LDL, cells were chased for the indicated periods of time at 37°C and the amounts of LDL internalized was measured as described in the Materials and Methods section. Values are means ± S.D. of triplicate incubations. The values for the zero-time binding to fibroblasts and HepG2 cells are 12.26 ± 0.7 and 7.9 ± 0.6 ng of LDL/mg of cell protein respectively, and are indicated on the ordinate by black and white arrows. This figure shows the results of one representative experiment out of two.

Rate of transport of ^{125}I-LDL from the early endosomes to the late endosomes or lysosomes in fibroblasts and HepG2 cells

To evaluate whether the delayed and less efficient degradation of LDL in HepG2 cells is due more specifically to an impaired transport of LDL from the early-endosomal compartment to the late-endosomal or lysosomal compartment, cells were incubated with ^{125}I-LDL at 18°C. At this temperature the degradation of LDL is inhibited, owing to an impairment in the dissociation of the internalized LDL from the receptor in the early-endosomal compartment [19] and to a block in endosome-lysosome fusion [20]. As a result, the cell-associated lipoproteins will accumulate in the early-endosomal compartment at this temperature.

In a time-course experiment at 18°C, we verified that in both fibroblasts and HepG2 cells indeed no detectable degradation of LDL occurred during the 4.5 h of incubation at 18°C (Fig. 4a). After that time the cells were washed in order to remove the unbound ligand and further incubated at 37°C (Fig. 4b). After the temperature shift to 37°C, in fibroblasts we observed a lag period of 30 min followed by a rapid appearance of degradation products into the medium. In HepG2 cells this lag period was about 90 min and the degradation of LDL appeared to occur at a slower rate than in fibroblasts.

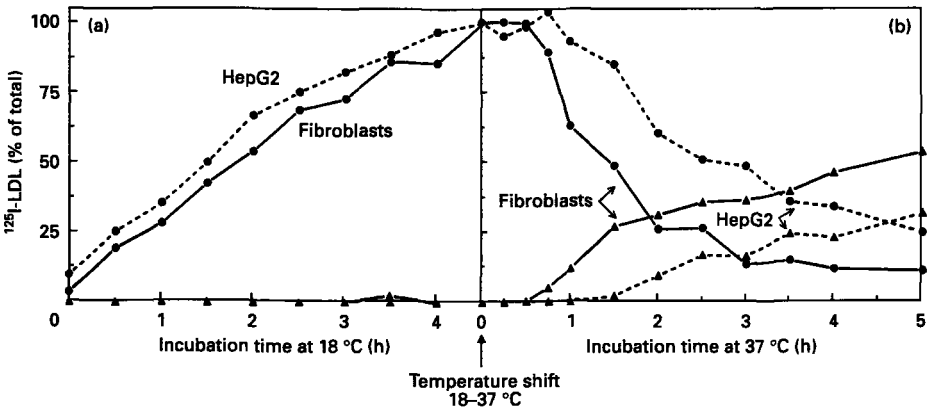

Figure 4. Intracellular processing of ^{125}I-LDL in fibroblasts (———) and HepG2 cells (----). Cells were preincubated at 18°C for 4.5 h in the presence of 10 μg of ^{125}I-LDL/ml with or without a 50-fold excess of unlabelled LDL and then chased for the indicated periods of time at 37°C. Receptor-mediated association (●) and degradation (▲) were measured. After 4.5 h at 18°C, less than 10% of the cell association appeared to be surface-bound, measured as that trypsin-releasable. Association and degradation are expressed as the percentage of the amount of ^{125}I-LDL that became cell-associated after 4.5 h at 18°C. The 100% values for association are 216 ± 21 and 114 ± 3 ng of LDL/mg of cell protein for fibroblasts and HepG2 cells respectively. This figure shows the results of one representative experiment out of two.

In a parallel experiment, the incubation for 4.5 h at 18°C was carried out in the presence of ^{125}I-TC-LDL, after which the temperature was shifted to 37°C and, at the indicated time points, cells were homogenized. The subcellular fractionation of cell homogenates shows

(Fig. 5, time 0 at 37°C) that, owing to their lower buoyant density, the early endosomes were separated from the lysosomes by Percoll-gradient centrifugation [16]. The late endosomes, having a density similar to that of the lysosomes [21], are recovered with the high-density fractions.

As shown in Fig. 5, at zero time all the label was found in the early-endosomal (light) fractions, whereas after 30 min of incubation at 37°C most of the label was found in the bottom fractions of the gradient. That these fractions contain lysosomal activity has been tested by measuring acid phosphatase activity as lysosomal marker [22].

As shown in Fig. 6, for both cell lines at zero time only about 5% of the total amount of radioactivity could be found in the high-density fractions, whereas the accumulation of ^{125}I-TC-LDL in the high-density fractions reached its maximum within almost 15 min.

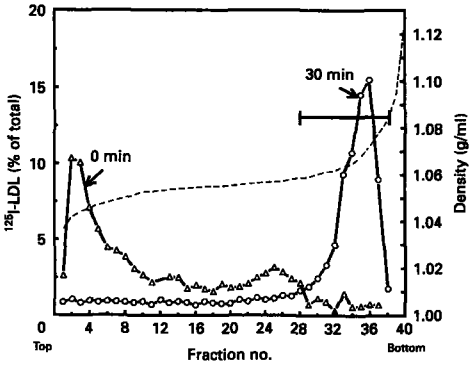

Figure 5. Subcellular distribution of ^{125}I-LDL. HepG2 cells were incubated for 4.5 h at 18°C with 10 μg/ml ^{125}I-TC-LDL in DMEM/1% HSA. After washing to remove unbound ligand, the cells were incubated at 37°C for various periods of time, after which they were homogenized and subjected to subcellular fractionation as described in the Materials and Methods section. The subcellular distribution of ^{125}I-TC-LDL after 0 (△) and 30 min (○) of incubation at 37°C is shown. The broken line (---) represents the density profile, and the horizontal bar indicates the fractions in which an activity above 0.4 unit of the lysosomal marker acid phosphatase was measured.

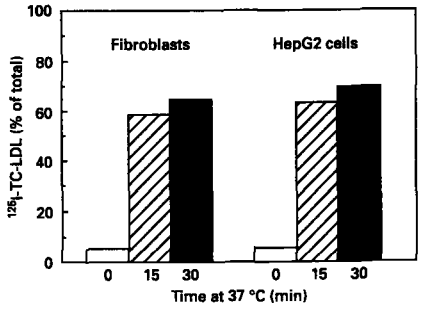

Figure 6. Accumulation of ^{125}I-TC-LDL in the late-endosomal and lysosomal fractions of fibroblasts and HepG2 cells as a function of time. Cell homogenates were fractionated on Percoll gradients as described in Fig. 5. For each time point the fractions representing the high-density fractions (30-38; see Fig. 5) were pooled and expressed as the percentage of the total amount of radioactivity present in the homogenate. The figure shows the results of one representative experiment out of two.

Discussion

Several studies have shown that HepG2 cells possess functional LDL receptors with properties similar to those of human fibroblasts [3]. However, some aspects of the mechanism regulating the LDL-receptor activity in both HepG2 cells and primary cultures

of human hepatocytes are likely to differ from those that have been characterized in fibroblasts and in cells derived from other peripheral tissues [2]. In this respect, we have demonstrated that both HepG2 cells [6] and freshly isolated human hepatocytes [7] are much less responsive to feedback regulation by LDL than are fibroblasts. In these studies it also appeared that the LDL-receptor activity in HepG2 cells and human hepatocytes was stimulated 2-3-fold by the presence of cholesterol acceptors such as heavy HDL, whereas in fibroblasts the LDL-receptor activity was almost insensitive to the presence of heavy HDL. Studies on the cellular cholesterol homoeostasis in relation to the LDL-receptor activity in HepG2 cells suggested that both the exogenously delivered (LDL) cholesterol and the endogenously synthesized cholesterol are primarily directed to a cholesteryl ester pool or, if present, to extracellular cholesterol acceptors, like heavy HDL, rather than to the regulatory free cholesterol pool involved in the regulation of the LDL-receptor activity [8].

In the present work we compared the processing of LDL in HepG2 cells and fibroblasts in more detail, reasoning that information about this pathway might help to explain the marked difference between the two cell lines in their ability to modulate the LDL-receptor activity in response to the presence of exogenous cholesterol.

Data reported in the literature [23,24] have revealed that in hepatocytes the LDL receptors are distributed diffusely on the basal surfaces, and only a small number are localized in endocytic vesicles. This is in sharp contrast with the distribution of LDL receptors in human fibroblasts, where most of the receptors are located in coated pits and in the membranes of the endocytic recycling pathway. Nevertheless, it has also been found that in liver cells the ligand-receptor complexes are only internalized after moving into coated pits [24]. It seems likely therefore that this difference in the cellular distribution of the LDL receptors could result in a slower internalization rate of LDL in hepatocytes. The present results show, however, that in HepG2 cells LDL is internalized at the same rate as in fibroblasts.

Our results show that the degradation of LDL in HepG2 cells appears to be severely impaired, being not only delayed but also inefficient, as expressed by the low ratio of degradation/cell association (Figures 1 and 2). A number of other studies concur with our findings of a relatively inefficient degradation of LDL in HepG2 cells. Edge et al. [25] also found that the binding and uptake of LDL in cultured human hepatocytes was similar to that seen in fibroblasts, whereas the degradation efficiency of LDL was lower than in fibroblasts. Kamps et al. [26] showed that in human parenchymal liver cells the LDL uptake is also not efficiently coupled to catabolism.

In our attempt to delineate the events responsible for the observed defect in the degradation of LDL in HepG2 cells, we measured the rate of distribution of LDL between the endosomes and the lysosomes in both cell lines. In these experiments cells were incubated at 18°C, since at this temperature no ligand-receptor dissociation and no fusion between endocytic vesicles and lysosomes occurs, and the ligand thus accumulates in the early-endosomal compartment [20]. When this approach was used, the difference in

LDL degradation between fibroblasts and HepG2 cells was still apparent (Fig. 4). Subsequently, the distribution of LDL between the endosomes and the lysosomes was measured with ^{125}I-LDL. Upon degradation of proteins, ^{125}I-TC remains attached to short peptide fragments, and in this form no release of label from the lysosomes occurs [27]. Using this experimental design, we did not observe any difference between both cell lines in the processing of LDL from the early-endosomal compartment to the late-endosomal or lysosomal compartment. This indicates that the dissociation of LDL from the receptor does not represent a rate-limiting step in the degradation of LDL. The method for subcellular fractionation used does not enable us to discriminate further between a pre-lysosomal and the lysosomal compartment. Therefore from our data we can conclude that in HepG2 cells either the fusion process between the late endosomes and the lysosomes is altered, or the degradation of LDL itself in the lysosomes is less efficient than in fibroblasts.

Several studies in fibroblasts [18], smooth-muscle cells [28], endothelial cells [29], A431 cells [30] and rat hepatocytes [31] have shown that the LDL-receptor activity decreases with increasing cell densities. Our results with HepG2 cells confirm this inverse correlation between cell density and LDL-receptor activity (Fig. 2). However, the lag period and the efficiency of the degradation, expressed as the ratio degradation/cell association, was not influenced by the cell density (Fig. 2).

The absence of a relationship between LDL-receptor activity and degradation efficiency is sustained by our previous observation that the stimulation of the LDL receptor in HepG2 cells by incubation with heavy HDL does not result in an increased efficiency of LDL degradation [6]. Similarly, ursodeoxycholic acid was found to enhance receptor-dependent LDL uptake to a similar extent to the degradation in isolated hamster hepatocytes [32].

A primarily direction of exogenously delivered LDL cholesterol and endogenously synthesized cholesterol to an intracellular cholesteryl ester pool or to extracellular cholesterol acceptors rather than to the regulatory free cholesterol pool involved in LDL-receptor activity has been reported [8]. With the present results, we argue that also a relatively low degradation efficiency of LDL in HepG2 cells may contribute to provide an explanation for the relatively weak down-regulation of the LDL-receptor activity in HepG2 cells on incubation of the cells with LDL.

Acknowledgements

This study was financially supported by a grant from the Dutch Heart Foundation (89.057 to P.L. and 87.025 to M.M.). Mrs Lenie de Pagter is gratefully acknowledged for taking care of cell cultures.

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Chapter 5

INEFFICIENT DEGRADATION OF TRIGLYCERIDE-RICH LIPOPROTEIN BY HEPG2 CELLS IS DUE TO A RETARDED TRANSPORT TO THE LYSOSOMAL COMPARTMENT

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J. Biol. Chem., in press.

Summary

Binding studies at 37°C showed that lipoprotein lipase treated very low density lipoproteins (LPL-VLDL) and very low density lipoproteins (VLDL), once taken up via the low density lipoprotein (LDL) receptor, are poorly degraded by HepG2 cells as compared with LDL. Determination of the initial endocytotic rate for LPL-VLDL and VLDL as compared to LDL shows that LPL-VLDL and VLDL are internalized at a similar rate as LDL. Incubation of cells with labelled LDL, LPL-VLDL and VLDL at 18°C for 4.5 hours resulted in the accumulation of these particles in the early endosomes, without subsequent transport to the lysosomes and degradation. After washing the cells and a temperature shift to 37°C, the labelled LDL present in the early endosomes is transported to the lysosomal compartment almost completely within 15 minutes. Strikingly, for LPL-VLDL and for VLDL, only about 50% or less of the label was moved to the lysosomal compartment within 45 minutes. However, once present in the lysosomes, VLDL and LPL-VLDL are degraded about 1.6-fold more rapidly than LDL. Retroendocytosis accounts for less than 10% of the internalized LDL, whereas a higher rate of retroendocytosis, up to 20% and 40%, respectively, was observed for LPL-VLDL and VLDL.

To evaluate the effect of the inefficient transport of VLDL and LPL-VLDL to the lysosomal compartment on cellular cholesterol homeostasis, acyl-CoA:cholesterol acyltransferase (ACAT) activity was measured. Incubation with 30 μg/ml of LDL induced a 2.5-fold increase in ACAT activity, whereas the incubation with similar amounts of both VLDL and LPL-VLDL failed to stimulate this enzyme.

We conclude that both a slower transport to the lysosomal compartment and a higher rate of retroendocytosis, possibly as the consequence of the longer residence-time in the early endosomes, are responsible for the poor degradation of VLDL and LPL-VLDL by HepG2 cells.

Introduction

Very low density lipoprotein (VLDL) are triglyceride-rich, apolipoprotein (apo) E and apoB100-containing, lipoprotein particles that are synthesized and secreted by the liver. After entering the bloodstream, VLDL particles interact with lipoprotein lipase (LPL), which catalyzes the hydrolysis of triglycerides. The resulting remnant particles are smaller, more dense and have an altered lipid and apolipoprotein composition, as compared with native VLDL particles (for review, see ref. 1). The VLDL remnants are further lipolysed and converted into Intermediate Density Lipoproteins (IDL) and, finally, Low Density Lipoprotein (LDL). During VLDL lipolysis, a fraction of the remnants is directly cleared from the plasma via hepatic LDL receptors, where apoE, the major protein constituent of these particles, acts as a ligand (2-6).

Many lipoprotein particles that contain apoE have several copies of this protein and are thought to react more avidly with the LDL receptor than LDL (7,8). A single lipoprotein particle containing several molecules of apoE could interact multivalently with a single LDL receptor; alternatively, lipoproteins containing several molecules of apoE may interact with more than one LDL receptor. In either case, particles that contain apoE in addition to apoB100 will bind to the LDL receptor with higher affinity than those that contain only one apoB100 molecule (9).

Recent studies by Tabas et al. (10) have shown that the multivalent binding of β-VLDL through apoE to the LDL receptor in mouse peritoneal macrophages leads to a divergent endocytotic pathway as compared to LDL. They found that LDL is rapidly targeted to perinuclear lysosomes near the centre of the cell, whereas, after its uptake, β-VLDL is localized in more distributed vesicles. This differential distribution was found to be coupled to a slower degradation of β-VLDL concomitant with a higher capability to stimulate acyl-CoA:cholesterol acyltransferase (ACAT).

In previous studies we found that the degradation of VLDL and LPL-treated VLDL, the latter taken as representative of VLDL-remnants, by HepG2 cells is extremely low as compared to that of LDL (unpublished observation). A low degradation efficiency of VLDL has also been described by other investigators (11). In the present study, we addressed the question as to whether this inefficient degradation might be due to an altered intracellular processing of these particles, possibly due to their multivalent binding via apoE. The present results clearly show that after internalization, the transport of VLDL as well as of LPL-treated VLDL to the lysosomal compartment is indeed severely retarded, whereas, once present in the lysosomes, these particles are catabolized about 1.6-fold more rapidly than LDL. In addition, we found that these lipoproteins fail to stimulate intracellular ACAT activity.

Materials and Methods

Materials

Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM, cell culture medium) were obtained from Flow Laboratories (Irvine, U.K.). Human serum albumin (HSA) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). $Na^{125}I$ (spec. act. 13.3 μCi/μl) was purchased from Amersham (Buckinghamshire, U.K.). Multiwell cell culture dishes were from Costar (Cambridge, MA, U.S.A.). Proteinase K was purchased from Boehringer Mannheim (Mannheim, Germany). Percoll (density 1.13 g/ml) was obtained from Pharmacia (Uppsala, Sweden).

Lipoproteins

LDL and VLDL were isolated from serum of normolipidemic donors by density gradient

ultracentrifugation according to Redgrave et al. (12). Lipoprotein lipase-treated VLDL (LPL-VLDL) were prepared by incubating total serum with lipoprotein lipase (LPL) purified from bovine milk (13), essentially as described before (5). Briefly, the amount of LPL added was equal to the amount necessary for hydrolysis of 50% of the triacylglycerols present in complete serum within 1 hour. The incubation was performed in the presence of 10% (w/v) fatty acid free human serum albumin (HSA) and Tris-HCl buffer (final concentration 0.1 M, pH 8.5) for 90 minutes at 37°C. To stop the reaction, the mixture was put on ice and solid KBr was added to adjust the solution to a density of 1.21 g/ml. LPL-VLDL, with density less than 1.019 g/ml, were then isolated by density gradient ultracentrifugation (12).

The lipoprotein preparations were immediately used for iodination by the ^{125}iodine monochloride method described by Bilheimer (14). After iodination, the lipoproteins were dialysed against phosphate buffered saline (PBS, pH 7.4) and stabilized with 1% (w/v) HSA. The specific activities ranged from 100 to 250 cpm/ng of protein. The stabilized ^{125}I-labelled lipoproteins were stored at 4°C and used within two weeks. With all labelled lipoproteins, less than 1% of the radioactivity was soluble in 10% (w/v) trichloroacetic acid (TCA). Whenever unlabelled lipoproteins were used, they were immediately stabilized after isolation with 1% (w/v) HSA followed by extensive dialysis against PBS and, subsequently, DMEM supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

Lipoprotein-depleted serum (LPDS) was obtained by ultracentrifugation of serum at a density of 1.21 g/ml followed by extensive dialysis of the infranatant against PBS and, subsequently, DMEM supplemented with penicillin and streptomycin.

β-VLDL was separated by sequential ultracentrifugation from the serum of cholesterol-fed rabbits (d < 1.006 g/ml) and extensively dialysed against PBS and, subsequently, DMEM supplemented with penicillin and streptomycin.

Cell culture

HepG2 cells were cultured in 25 cm^2 flasks in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 20 mM Hepes, 10 mM $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in equilibration with 95% air/5% CO_2. Six to seven days prior to each experiment, cells were seeded in 2 cm^2 multiwell dishes.

Twenty-four hours before the assays, the cells were washed with DMEM-1% HSA and further incubated with DMEM containing 10% LPDS (v/v) instead of FCS.

Receptor-mediated cell association and degradation

Receptor-mediated cell association and degradation were measured essentially as described previously (5,15).

Each experiment was started by washing the cells three times in DMEM-1% HSA. Thereafter, cells were incubated in the same medium with the addition of 20 μg/ml of labelled lipoproteins, in the presence or absence of a 30-fold excess of unlabelled LDL. Temperature and duration of the respective incubations are described in the text and figure legends. After incubation with labelled lipoproteins, cells were cooled to 0°C. Degradation

was measured exactly as described (16) and, after removal of the incubation medium, the cells were washed extensively (17). To measure total cell association, the washed cells were dissolved in 0.2 M NaOH and an aliquot of the cell lysate was counted for radioactivity. Another aliquot of the cell lysate was used for protein determination according to Lowry et al. (18).

Values for the receptor-mediated cell association and degradation were calculated by subtracting the amount of labelled lipoprotein that was cell-associated or degraded in the presence of a 30-fold excess of unlabelled LDL (nonspecific binding) from the amount of labelled lipoprotein that was cell-associated or degraded in the absence of an excess of unlabelled LDL (total binding).

Initial rate of endocytosis

To measure the initial rate of endocytosis, cells were incubated for the indicated periods of time at 37°C in DMEM-1% HSA with the addition of 20 μg/ml of labelled lipoproteins, in the presence or absence of a 30-fold excess of unlabelled LDL. Cells were then washed extensively and further incubated with 0.5 mg/ml Proteinase K, 1 mM EDTA in PBS for 1 hour at 0°C. The protease activity was then neutralized by adding the same volume of PBS containing 1 mM EDTA and 1 mM phenyl-methyl-sulphonylfluoride (PMSF). The detached cells were collected by centrifugation at 150 g for 5 minutes at 0°C. The radioactivity released into the buffer represents the amount of ^{125}I-labelled lipoprotein bound to the cell membrane. The radioactivity that remains cell-associated represents the amount of ^{125}I-labelled lipoprotein internalized.

Values for the receptor-mediated binding and internalization were calculated by subtracting the amount of labelled lipoprotein that was bound or internalized in the presence of a 30-fold excess of unlabelled LDL from the amount of labelled lipoprotein that was bound or internalized in the absence of an excess of unlabelled LDL.

Measurement of intracellular transport of lipoproteins

Subcellular fractionation of HepG2 cells was performed by Percoll density gradient centrifugation as described (19). Cells, seeded in 60 or 100 mm dishes, were incubated in the presence of 20 μg/ml of ^{125}I-labelled LDL, LPL-VLDL or VLDL. Temperature and duration of the respective incubations are described in the text and figure legends. After incubation with the labelled lipoproteins, cells were cooled to 0°C. The incubation medium was removed and degradation measured as described (16). Cells were then washed extensively with 0.28 M sucrose, 2 mM $CaCl_2$, 0.01 M Tris-HCl pH 7.6, and scraped from the dishes with a rubber policeman in the same buffer (1 ml/dish). Then, cells were homogenized in a Dounce homogeniser by 20 complete strokes with a tight fitting pestle. The homogenates were centrifuged at 280 x g for 10 minutes in order to remove remaining intact cells. A 80% (v/v) Percoll solution and homogenization buffer were added to the supernatants to a final Percoll concentration of 20% and to a final volume of 12 ml. After thorough mixing, the samples in Percoll were placed in cellulose nitrate tubes fitting a 50Ti rotor

(Beckman) and centrifuged at 10,000 x g for 45 minutes. Fractions of ~ 0.3 or 0.5 ml were collected by aspiration from top to bottom and the radioactivity in each sample was counted. The density of each fraction was measured in a PAAR-DMA-45 density meter equipped with a DMA-602M small sample cell (~ 170 μl). The distribution of the lysosomal marker (acid phosphatase) was measured by the method of Torriani (20).

Measurement of intracellular cholesterol esterification (ACAT activity)

Cholesterol esterification was measured by determining the incorporation of [1-^{14}C]-oleic acid into labelled cholesteryl oleate, essentially as described (21), with some minor modifications. Cells cultured in 10 cm^2 wells were first preincubated for 20 hours in culture medium containing 10% lipoprotein deficient serum (LPDS) and then with the indicated amounts of lipoproteins for 4 hours. Subsequently, to 2 ml of incubation medium, 100 μl of a 1 mM solution of [^{14}C]-oleate (2340 dpm/nmol) complexed to albumin was added and cells were incubated for another 2 hours at 37°C. Cells were then washed four times with ice-cold PBS and harvested in 1 ml by scraping. After addition of [^{3}H]-cholesterol (60,000 dpm/sample), as an internal standard, lipid extraction as described by Bligh and Dyer (22) was performed. Lipids were analyzed by thin layer chromatography on pre-coated silica plates. The developing solvent was composed of chloroform/methanol (98:2) (v/v) first, followed by chloroform/hexane (45:65) (v/v). The lipid spots were detected by autoradiography, scraped off and counted for radioactivity in a Packard 1900CA Tri-Carb liquid scintillation analyzer equipped with software validated for ^{14}C/^{3}H double labelled samples. Recovery of the internal standard was 65-85%.

Results

Time course of receptor-mediated association and degradation of LDL, LPL treated VLDL and VLDL by HepG2 cells

The time-course of receptor-mediated association and degradation of labelled LDL, LPL-VLDL and VLDL at 37°C are shown in Fig. 1. For all three lipoprotein samples, the cell-association increased progressively over the first three hours, before a plateau is reached. The degradation started after a lag period of 60 to 90 minutes and proceeded at a slower rate in case of both LPL-VLDL and VLDL, as compared with LDL. When the degradation efficiency is calculated as the amount of lipoprotein degraded relative to the amount of lipoprotein that became cell-associated (Fig. 2), it is apparent that after 5 hours of incubation the degradation efficiency of LPL-VLDL and VLDL is only 50% and 20%, respectively, of that of LDL.

We reasoned that more information about the intracellular processing of LPL-VLDL and VLDL might help explaining this difference in degradation efficiency. Therefore, the next experiments were designed to investigate whether the reduced degradation efficiency of LPL-

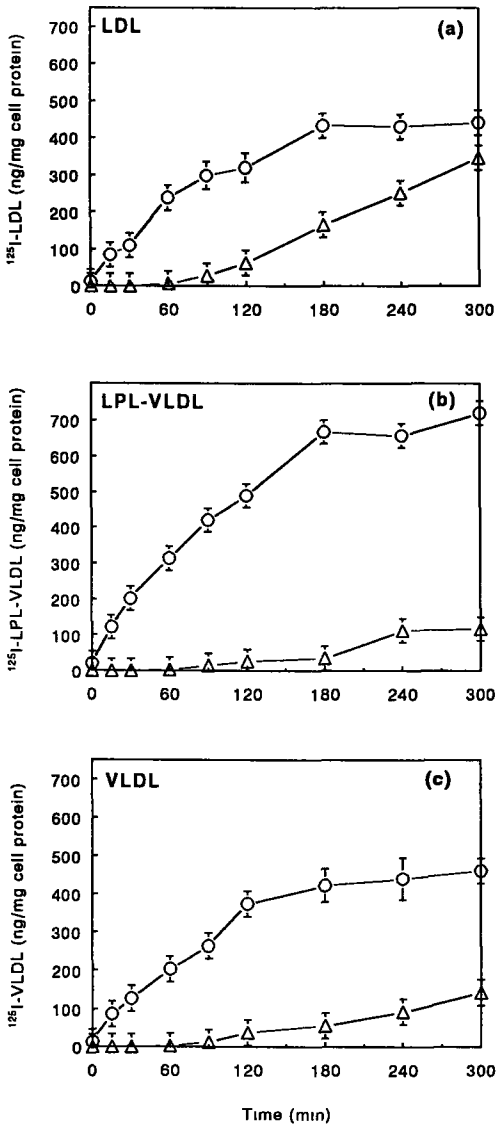

Figure 1. Time course at 37°C of the receptor-mediated association (○) and degradation (△) of LDL (A), LPL-VLDL (B) and VLDL (C) in HepG2 cells. Cells were incubated with 20 μg/ml of ^{125}I-LDL or ^{125}I-LPL-VLDL or ^{125}I-VLDL ± 30-fold excess of unlabelled LDL at 37°C for the indicated periods of time. Thereafter, the receptor-mediated association and degradation were measured as described in Materials and Methods. Values are means ± S.D. of triplicate incubations.

VLDL and VLDL was due to: (*i*) a lower rate of endocytosis, (*ii*) a less efficient transport of the apoE-binding lipoproteins from the early endosomal compartment to the lysosomal compartment or (*iii*) an impairment in the lysosomal degradation itself.

Initial rate of endocytosis of surface-bound LDL, LPL-VLDL and VLDL by HepG2 cells

The initial endocytotic rate of LDL, LPL-VLDL and VLDL was studied as described by Wiley and Cunningham (23) (Fig. 3). Cells were incubated with 20 μg/ml of labelled lipoproteins. At the specified time points, cells were extensively washed and the ratio of

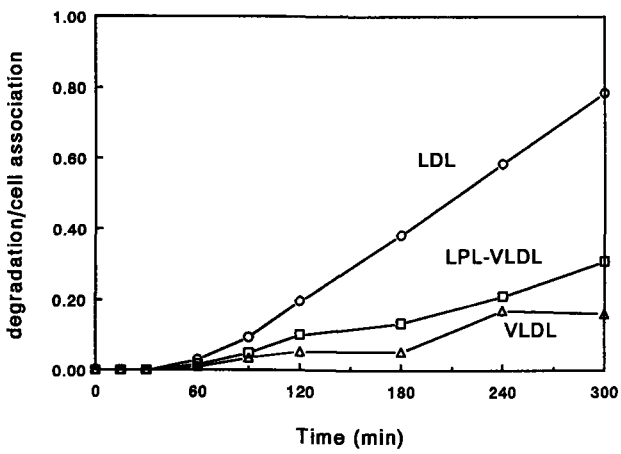

Figure 2. Degradation efficiency of LDL (○), LPL-VLDL (□) and VLDL (△). Values are calculated from the results presented in Fig. 1 as ratio degradation/association.

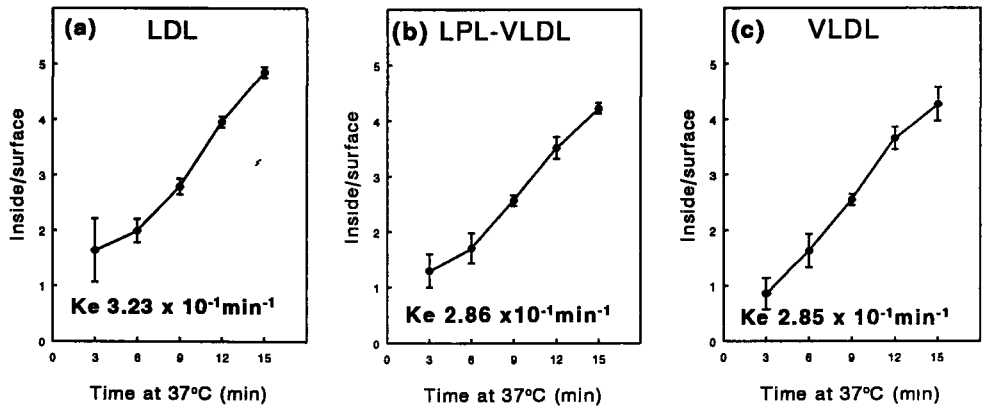

Figure 3. Initial endocytotic rate of LDL (A), LPL-VLDL (B) and VLDL (C) in HepG2 cells. Cells were incubated at 37°C with 20 μg/ml of ^{125}I-LDL, ^{125}I-LPL-VLDL or ^{125}I-VLDL ± 30-fold excess of unlabelled LDL. At the indicated time points cells were quickly washed at 0°C, and the ratio of radioactivity internalized to that bound to the surface was determined as outlined in Materials and Methods. Values are means ± S.D. of triplicate incubations. Ke indicates the endocytotic rate constant.

radioactivity associated with the interior of cells (internalized) to that associated with the surface (bound) was measured. Figure 3 shows that the rate of endocytosis of LPL-VLDL and VLDL is similar to that of LDL. We hypothesize therefore that LPL-VLDL and VLDL, once internalized at a normal rate, (*i*) cannot be further transported to the lysosomal compartment, or (*ii*) they cannot be degraded in the lysosomes either due to an impairment in the late endosome-lysosome fusion or to a defect in the lysosomal degradation itself.

Intracellular processing and rate of retroendocytosis of LDL, LPL-VLDL and VLDL
To evaluate whether the transport of LPL-VLDL and VLDL from the early endosomal compartment to the late endosomal or lysosomal compartment is impaired, cells were first incubated with labelled lipoproteins for 4.5 hours at 18°C. At this temperature, it has been demonstrated that degradation of LDL is inhibited owing to an impairment in the dissociation of the internalized LDL from the receptor (24) and to a block in endosome-lysosome fusion (25). As a result, the cell-associated lipoproteins will accumulate in the early endosomal compartment, without being degraded (26).

After the incubation in the presence of labelled LDL, LPL-VLDL or VLDL at 18°C, cells were washed in order to remove the unbound ligand and further incubated at 37°C for the indicated periods of time (Fig. 4). With LPL-VLDL and VLDL the major portion of the initial amount of label accumulated in the endosomes is still cell-associated after 5 hours at 37°C (Fig. 4B and 4C), whereas for LDL (Fig. 4A) about 70% of the internalized LDL is degraded within 5 hours after the temperature shift from 18°C to 37°C. For LDL, the decrease in cell association is fully complementary to the amount of LDL degraded. This implies that all intracellularly present LDL is released, after degradation. Strikingly, for LPL-VLDL and VLDL the sharp decline of the cell association curve in the first hour of incubation after the temperature shift from 18°C to 37°C suggests that part of the intracellularly-accumulated particles are excreted as intact particles into the medium, thus escaping the degradation route (retroendocytosis), a process which has been found to account for up to 10% of the internalized LDL (27). To further investigate this possibility, retroendocytosis was calculated from the data reported in Fig. 4. Indeed, we found that retroendocytosis (Fig. 4, broken line) accounts for less than 10% of the initially internalized LDL, whereas up to 20% and 40% of LPL-VLDL and VLDL, respectively, appear to be released intact into the medium.

These data indicate that the inefficient degradation of LPL-VLDL and VLDL is the result of the combined effect of an impaired process downstream the early endosomal compartment, and of retroendocytosis that diverts a substantial amount of the internalized LPL-VLDL and VLDL from the intracellular processing.

Rate of transport of LDL, LPL-VLDL and VLDL from the early endosomes to the lysosomes
To investigate as to whether LPL-VLDL and VLDL are either retained in the sorting endosomes or normally delivered to the lysosomal compartment, but not further degraded, cells were incubated for 4.5 hours at 18°C in the presence of labelled lipoprotein, followed by a temperature shift to 37°C and homogenization at the indicated time points. Thereafter, cell homogenates were subcellularly fractionated (Fig. 5) (19). Due to their difference in buoyant density, the early and sorting endosomes (top fractions) were separated from the lysosomal fractions (bottom fractions) by Percoll-gradient centrifugation. The late endosomes, having a density similar to that of the lysosomes (28), are recovered with the high density fraction. Since a further discrimination between the late endosomal and the lysosomal compartment is not feasible with the method selected for subcellular fractionation, we will

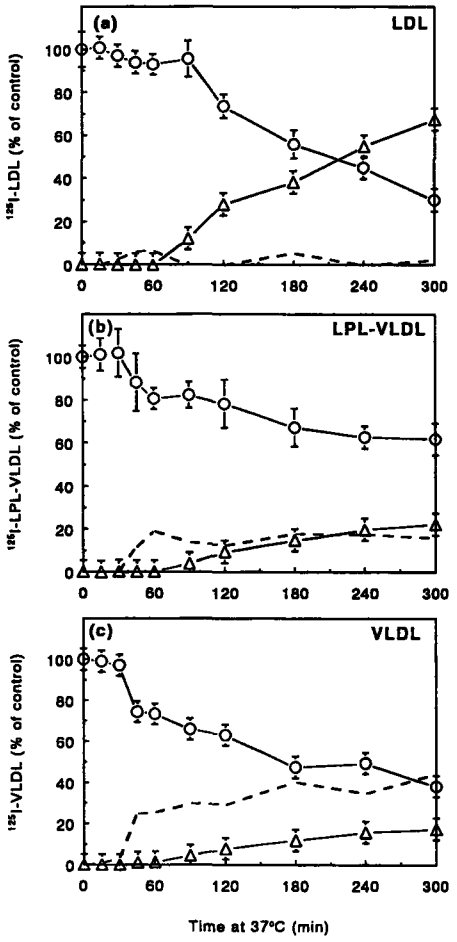

Figure 4. Intracellular processing and rate of retroendocytosis of LDL (A), LPL-VLDL (B) and VLDL (C). Cells were preincubated at 18°C for 4.5 hours in the presence of 20 μg/ml of ^{125}I-labelled lipoproteins ± 30-fold excess of unlabelled LDL and then chased for the indicated periods of time at 37°C. Receptor-mediated association (○) and degradation (△) were measured. The amount of lipoprotein associated at time 0 was taken as 100% (control value). The 100% values of the cell-association at time 0 at 37°C are 74 ± 8, 127 ± 4, 62 ± 4 ng/mg cell protein for LDL, LPL-VLDL and VLDL, respectively. At each time point the retroendocytosis rate for LDL, LPL-VLDL and VLDL (—) was calculated according to the formula: retroendocytosis = 100% (% lipoprotein cell associated + % lipoprotein degraded). Values are means ± S.D. of triplicate incubations.

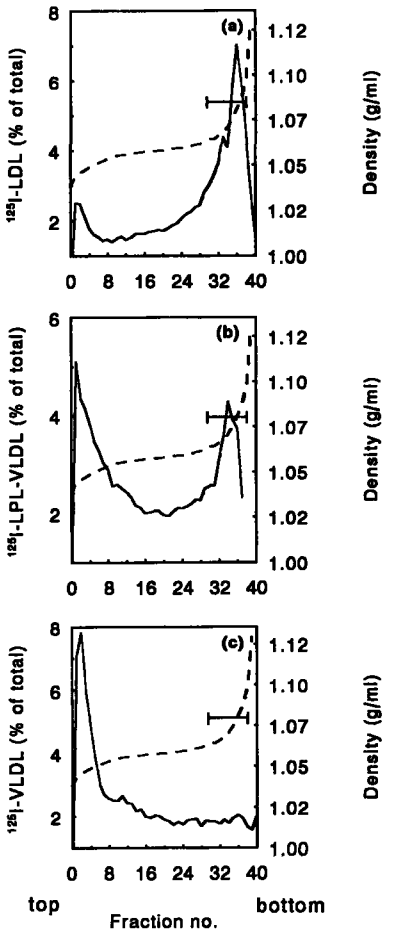

Figure 5. Subcellular distribution of LDL (A), LPL-VLDL (B) and VLDL (C) in HepG2 cells. Cells were incubated for 4.5 hours at 18°C with 20 μg/ml ^{125}I-labelled lipoprotein in DMEM-1% HSA medium. After washing to remove unbound ligand, the cells were incubated at 37°C for 15 minutes after which the cells were homogenized and subjected to subcellular fractionation as described in Materials and Methods. Fractions were measured for radioactivity. The dotted line represents the density profile of the gradient; the horizontal bar indicates the samples representing the lysosomal fractions as evaluated by acid phosphatase activity measurement.

refer to the high density fractions as those representing the late endosomal-lysosomal compartment. Figure 5 shows the distribution of label in the gradient fractions for each lipoprotein tested at one time point (15 minutes) after the temperature shift. With LDL (Fig. 5A), after 15 minutes at 37°C, almost all the radioactivity was found in the high density bottom fractions, which represent the late endosomal fractions and lysosomal fractions. The latter were identified by the presence of acid phosphatase activity (horizontal bar). Strikingly, LPL-VLDL, and even more dramatically VLDL, move much more slowly to the bottom fractions upon incubation at 37°C. After 15 minutes at 37°C, more than 50% of LPL-VLDL (Fig. 5B) and almost all VLDL (Fig. 5C) was still present in the light, early endosomal fractions. The rate of accumulation of the labelled lipoproteins in the high density fractions at different time points is summarized in Fig. 6. Within 15 minutes after the temperature shift to 37°C, the intracellular trafficking of LDL towards the late endosomal-lysosomal compartment was nearly complete, while for LPL-VLDL and VLDL, even after 45 minutes, the entire process towards the late endosomal-lysosomal compartment has not been completed. When the presence of labelled particles in the high density fractions was measured at longer time intervals (up to 90 minutes), the rate of accumulation declined, as a result of the increase in the amount of lipoprotein degraded (not shown). Apparently, LPL-VLDL and VLDL are much more slowly transported to the late endosomes or lysosomes than LDL.

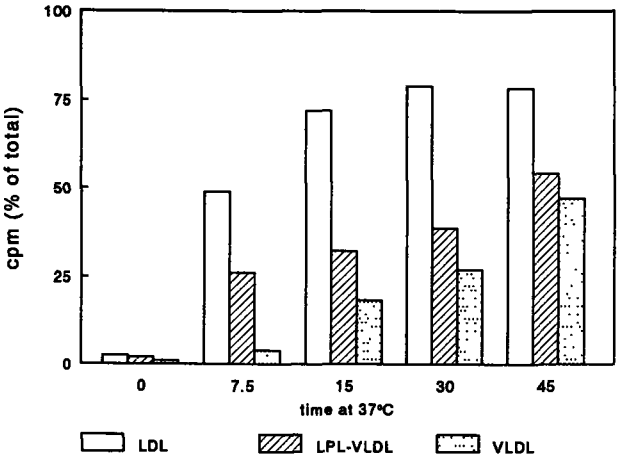

Figure 6. Accumulation of LDL, LPL-VLDL and VLDL in the late endosomal and lysosomal fractions as a function of time. Cell homogenates were fractionated on Percoll gradients as described in Fig. 5. For each time point the fractions representing the lysosomal fractions (fractions 30-38, see Fig. 5) were pooled and expressed as the percentage of the total amount of radioactivity present in the homogenate. Open bars represent LDL; hatched bars represent LPL-VLDL and dotted bars represent VLDL.

Rate of degradation of LDL, LPL-VLDL and VLDL in the lysosomes

Figure 5 indicates that, although at a slower rate, a substantial amount of LPL-VLDL and VLDL reaches the lysosomes. This would suggest that next to a slower transport to the lysosomes, a slower turnover of LPL-VLDL and VLDL in the lysosomes might also contribute to the overall effect of a sluggish catabolism of these particles. To verify this hypothesis, cells were incubated with labelled LDL, LPL-VLDL and VLDL at 37°C for 5

Table 1. Turnover rate of LDL, LPL-VLDL and VLDL in lysosomes. Cells were incubated in the presence of 20 μg/ml of ^{125}I-labelled LDL, LPL-VLDL or VLDL at 37°C for 5 hours. Cells were then rapidly washed at 37°C with pre-warmed medium and further incubated for 30 minutes at 37°C in the presence of the same amount of labelled lipoproteins as in the previous incubation. Cells were cooled to 0°C, washed extensively, homogenized and subjected to subcellular fractionation as described in Materials and Methods.

	lipoprotein degraded[a]/lipoprotein present in the lysosomes[b]
LDL	1.064 ± 0.063[c]
LPL-VLDL	1.61 ± 0.019
VLDL	1.59 ± 0.024

[a] Expressed in ng/mg cell protein per 30 minutes.
[b] Measured as radioactivity present in the high density fractions of Percoll gradients and expressed as ng/mg cell protein.
[c] Values are means ± S.D. of duplicate incubations.

hours. At this time point cells are assumed to have reached a steady-state, as evaluated from the cell association curve shown in Fig. 1. Cells were then rapidly washed at 37°C with pre-warmed medium and further incubated for 30 minutes at 37°C in the presence of the same amount of labelled lipoproteins as in the previous incubation. Medium was collected for measuring degradation, after which cells were cooled to 0°C, washed extensively, homogenized and subjected to subcellular fractionation. The rate of degradation was calculated as the ratio of the amount of lipoprotein degraded in 30 minutes over the amount of lipoprotein present in the lysosomal fractions at steady-state. The results presented in Table 1 show that, once present in the lysosomes, LPL-VLDL and VLDL are degraded even more efficiently than LDL. Thus an impaired lysosomal degradation itself is not responsible for the observed low degradation efficiency of VLDL and LPL-VLDL by HepG2 cells (Figs. 1 and 2).

Cellular cholesterol esterification (ACAT activity)

In order to determine if there was a correlation between the retarded transport of LPL-VLDL and VLDL to the late-endosomal or lysosomal compartment and the potency of these particles to stimulate ACAT, ACAT activity was measured after incubation of HepG2 cells with either LDL, LPL-VLDL, VLDL or rabbit β-VLDL. In macrophages, β-VLDL are known to be a much more potent stimulator of ACAT than LDL, although this effect is not due to a greater delivery to the cell of β-VLDL cholesterol (29). As shown in Fig. 7, after 6 hours of incubation with 30 μg/ml of LDL, a 2.5-fold increase of the enzyme activity was obtained, as compared to the control level of ACAT activity in HepG2 cells. Similar amounts of β-VLDL stimulated ACAT up to 5-fold. LPL-VLDL and VLDL did not influence cellular ACAT activity at all. The same results were obtained when cells were incubated for a prolonged time (20 hours instead of 6 hours) and in the presence of higher amounts of

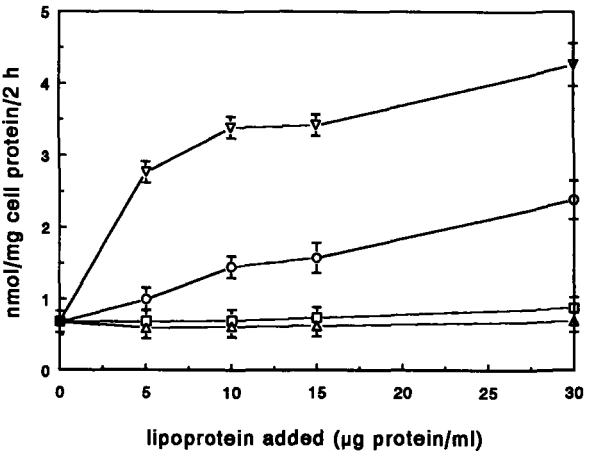

Figure 7. Cellular cholesterol esterification (ACAT activity) upon incubation with different lipoprotein fractions. Cells were incubated for 6 hours with DMEM, 10% LPDS containing the indicated amounts of rabbit β-VLDL (▽), LDL (○), LPL-VLDL (□) and VLDL (△). During the last 2 hours of the 6 hours incubation at 37°C, to the cells [^{14}C]-oleate (2340 dpm/nmol) was added. At the end of the 6 hours incubation, the cells were assayed for cholesteryl [^{14}C]-oleate content as described in Materials and Methods. Values are means ± S.D. of triplicate incubations.

lipoprotein (up to 150 μg of lipoprotein protein/ml). Results similar to those presented in Fig. 7 were obtained when the amount of lipoprotein added was expressed as μg cholesterol/ml. The poor ability of LPL-VLDL and VLDL to stimulate ACAT is in accordance with the retarded transport of these particles to the late-endosomal/lysosomal compartment.

Discussion

In the present study we have shown that normal VLDL and lipolyzed VLDL, taken as representative of VLDL remnants, once bound and taken up by the LDL receptor in HepG2 cells, are poorly degraded as compared to LDL. A low degradation efficiency has also previously been reported for both VLDL (11) and VLDL remnants (30). In the latter study, the authors propose that either a rapid dissociation of IDL/receptor complexes at the cell surface might take place, prior to internalization, or IDL might be internalized but a major fraction recycles back to the cell surface (retro-endocytosis), possibly together with the receptor protein, thus preventing the routing to the lysosomes. Our present data rule out the first hypothesis, clearly showing that the rate of endocytosis of VLDL and LPL-VLDL is similar to that of LDL (Fig. 3). In case of LPL-VLDL and VLDL, retroendocytosis accounts to a varying extent (from 20% up to 40%) for the amount of label which does not reach the lysosomal compartment. This suggests that a substantial amount of lipoproteins, especially VLDL, is diverted from the routing to the lysosomes and is excreted as intact particles into the medium (Fig. 4). In addition, the fraction of LPL-VLDL and VLDL that does not undergo retroendocytosis is only slowly transported to the lysosomal compartment, whereas, once present in the lysosomes, LPL-VLDL and VLDL are degraded 1.6-fold more rapidly than LDL. This implies that LPL-VLDL and VLDL reside for a longer time than LDL in the early endosomal compartment, thus increasing the probability that they return to the cell

surface by retroendocytosis.

Therefore, both a slower transport to the lysosomal compartment and a higher rate of retroendocytosis, possibly as the consequence of the longer residence-time in the early endosomes, are responsible for the poor degradation of VLDL and LPL-VLDL by HepG2 cells.

The retarded intracellular routing of these particles might be the result of the polyvalent binding of apoE in VLDL and LPL-VLDL to the receptor. Recently, such a mechanism has been postulated for β-VLDL in mouse peritoneal macrophages (10). It is hypothesized that the high-affinity polyvalent apoE binding to the LDL receptor results in a greater resistance to the acid-mediated release of the ligand from the receptor. If this is the case, the rate-limiting step in the processing of VLDL and LPL-VLDL indeed would take place in the sorting endosomes, thus raising the question of the fate of the receptors bound to the ligand. Previous studies have indicated that receptor cross-linking can block ligand-receptor recycling (31,32), sometimes triggering the delivery of the multivalent-bound receptors to the lysosomes for degradation. Our results, however, cannot discriminate between the two possibilities that either the receptor is relatively slowly recycled back to the plasma membrane or, eventually, partly degraded in the lysosomes.

In order to verify the effect of the slower processing and degradation of VLDL and LPL-VLDL on cellular cholesterol homoeostasis, we measured ACAT activity, which is known to be a sensitive measure for the amount of cholesterol in the regulatory cellular cholesterol pool. Eisenberg et al. (11) and Krul et al. (33) have found that incubation of cells with VLDL did not lead to a stimulation of ACAT activity. Our results are in line with their results. Both VLDL and LPL-VLDL were not able to stimulate the intracellular cholesterol-ester synthesis (Fig. 7). In contrast with this, Krul et al. (33) and Evans et al. (34) showed that VLDL isolated from hypertriglyceridemic (or type IV) subjects (HTG-VLDL) was a potent stimulator of ACAT. They showed that HTG-VLDL contains more apoE and more cholesterol per particle. However, a higher cholesterol content per HTG-VLDL particle, as compared with normal VLDL, cannot explain the discrepancy between their results and our results regarding the stimulation of ACAT activity. We observed that the cholesterol and apoE content (expressed as ratio cholesterol to triglycerides and apoE to apoB100, respectively) of the LPL-VLDL particles used in our study are in the same order of magnitude as that of the HTG-VLDL used by Evans et al. (results not shown) (34). Furthermore, expressing the amount of lipoprotein added in Fig. 7 as the amount of cholesterol added, instead of the amount of protein, did not considerably change the results shown.

Recently, Xu and Tabas (35,36) have found that in macrophages the cellular cholesterol levels first have to reach a critical threshold of about 25% above the basal level, before ACAT activity is stimulated. If the same 25% increase in cellular cholesterol level is required in HepG2 cells in order to stimulate ACAT activity, our results indicate that, under the conditions applied, VLDL and LPL-VLDL do not increase the ACAT substrate pool enough for exerting an effect on the ACAT activity. Since the amount of uptake of VLDL

and LPL-VLDL is comparable with the uptake of LDL (Fig. 1), also when based on the amount of cholesterol uptake (not shown), we conclude from our results that the ACAT substrate pool is supplied with lipoprotein-derived cholesterol only after the lipoproteins have been degraded. Hence, the cellular degradation of VLDL and LPL-VLDL is too inefficient to increase cellular cholesterol esterification.

A low degradation efficiency of VLDL and LPL-VLDL would also imply a relatively poor down-regulation of the LDL receptor activity upon incubation of cells with these lipoproteins. Epidemiological studies suggest that the down-regulation of the LDL receptor activity in the liver by VLDL and VLDL-remnants depends, at least partly, on the polymorphism of apoE (37-39). Whether, besides affecting the binding of the lipoproteins to the receptor, apoE polymorphism also interferes with the efficiency of cellular degradation of VLDL or VLDL-remnants, as a consequence of a retarded intracellular transport to the lysosomal compartment, is currently under investigation.

Acknowledgements

This study was financially supported by grants from the Dutch Heart Foundation (##89.057 to P.L. and 87.025 to M.M.). Mrs. Lenie de Pagter is gratefully acknowledged for taking care of cell cultures. We thank Mrs. Elly de Wit for expert technical assistance.

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Chapter 6

LOW DENSITY LIPOPROTEIN RECEPTOR INTERNALIZES LOW DENSITY AND VERY LOW DENSITY LIPOPROTEINS WHICH ARE BOUND TO HEPARAN SULPHATE PROTEOGLYCANS VIA LIPOPROTEIN LIPASE

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J. Biol. Chem. 1993; 268: 9369-9375 (reprinted with permission).

Summary

It has previously been shown that lipoprotein lipase (LPL) enhances the binding of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) to HepG2 cells and fibroblasts, up to 80-fold. This increase in binding is LDL receptor-independent and is due to a bridging of LPL between extracellular heparan sulphate proteoglycans (HSPG) and the lipoproteins. In the present paper, we show that preincubation of the cells with LPL, followed by washing prior to the binding experiment, increased binding to the same extent as occurs when the binding is performed in the presence of LPL. This indicates that the formation of a complex of LPL with the lipoproteins is not a prerequisite of binding. Binding curves and Scatchard analyses reveal that both the number of binding sites and the affinity of the binding is increased 20- to 30-fold by the addition of 3.4 μg/ml of LPL. The addition of LPL also resulted in an enhanced uptake and subsequent lysosomal degradation of both LDL and VLDL when compared with binding, although to a lesser extent (up to 25-fold, when measured after 5 hours at 37°C). Strikingly, enhanced uptake did not occur in LDL receptor-negative fibroblasts. In addition, down-regulation of the LDL receptor activity by preincubation of the cells for 48 hours with either LDL or β-VLDL, resulted in a parallel decrease in the uptake of LPL-mediated HSPG-bound LDL, whereas the LPL-mediated binding itself was not diminished. These observations indicate that the uptake of LPL-mediated HSPG-bound LDL and VLDL mainly proceeds via the LDL receptor. Binding of labeled LDL to the cells at 4°C for two hours followed by a chase period at 37°C, revealed that, in absolute terms, the initial rate of internalization of HSPG-bound LDL is comparable to that of LDL receptor-bound LDL (0.58 and 0.44 ng/minute/mg cell protein, respectively). We conclude that in LDL receptor-positive cells the LPL-mediated binding of LDL and VLDL to HSPG is followed by internalization of the lipoproteins mainly through the rapid process of the classical LDL receptor recycling system, whereas only a minor portion is internalized via the much slower process of HSPG uptake.

Introduction

In the circulation, chylomicrons and very low density lipoproteins (VLDL) are partly lipolysed through the action of endothelium-bound lipoprotein lipase (LPL). The resulting chylomicron- and VLDL-remnants are rapidly taken up after binding to hepatic receptors, mainly through one of their major protein constituents apolipoprotein E (apoE). Liver cells possess two different types of lipoprotein receptors. One receptor recognizes both apoB and apoE and is designated B,E receptor or LDL receptor. The other receptor recognizes only apoE and is designated as apoE or remnant receptor (1). The LDL receptor related protein (LRP) described by Herz et al. (2) appeared to be a potential candidate for the remnant receptor (3,4) and was observed to be structurally identical to the α_2-macroglobulin receptor

(5). The LRP proved to be a multifunctional receptor. It is not yet certain whether the LRP actually is the remnant receptor.

Chylomicrons have been reported to be taken up exclusively through the remnant receptor (6), although the involvement of the LDL receptor in chylomicron remnant clearance has also been suggested (7). Uptake of VLDL and VLDL remnants by the liver is reported to be mediated exclusively through the LDL receptor (8,9), although others have found that the remnant receptor is also involved in the processing of these lipoproteins (10). Harkes et al. (11) and De Water et al. (12) have shown that in the rat liver almost all β-VLDL is taken up via a putative remnant receptor on parenchymal liver cells which is different from the liver α_2-macroglobulin recognition site (13).

Recently, it has been found that the binding of chylomicrons and β-VLDL to either HepG2 cells or LDL receptor-negative fibroblasts was strongly increased when bovine or human LPL was added to the medium (14). It has been suggested that the LPL protein stimulates the interaction of apoE with LRP. Recently, we found that the stimulating effect of LPL on lipoprotein binding also holds for apoE-free LDL (15).

In addition, we provided evidence that neither the LDL receptor nor the LRP is responsible for the LPL-mediated stimulation of the binding of LDL and VLDL. We found that the enhancing effect of LPL on the binding of these lipoproteins could be prevented by pre-incubating the cells with heparinase, which is known to prevent high affinity binding of LPL to heparan sulphate proteoglycans (HSPG) (16,17). This led us to conclude that the stimulation of the binding is caused by the bridging of LPL between proteoglycans present on the plasma membrane and the lipoproteins.

Recently, Williams et al. (18) have also reported that LPL enhances the binding of apoB100-rich lipoproteins, such as LDL and Lp(a), via binding to HSPG. They found that the LPL-mediated cell association of Lp(a) is completely LDL receptor-independent, whereas the subsequent degradation of this lipoprotein is partly LDL receptor-dependent. In the case of LDL and nascent apoB-containing lipoproteins, the LPL-mediated cell association and degradation appeared both to be independent of LDL receptor activity. Rumsey et al. (19) also reported that the LDL receptor is not involved in the LPL-mediated binding and uptake of LDL by both fibroblasts and THP-1 macrophages. With the results presented in this paper we obtained strong evidence that the LDL receptor is responsible for the major part of the uptake of (LPL-mediated) HSPG-bound LDL and VLDL, whereas only a minor part of HSPG-bound LDL and VLDL is directly internalized, thus without the LDL receptor. We also show that the rate of internalization of HSPG-bound LDL via the LDL receptor is comparable to that of LDL which is directly bound to the LDL receptor.

Materials and methods

Lipoproteins

Blood was obtained from healthy volunteers, after an overnight fast. Serum was separated

from the cells by centrifugation at 500 g for 15 min at room temperature. LDL (density 1.035-1.06 g/ml), VLDL (density d < 1.019 g/ml) and heavy HDL (density 1.16-1.20 g/ml) were isolated by ultracentrifugation, using the procedure as previously described (8). β-VLDL was obtained from fasted serum of male Wistar rats that were maintained on a cholesterol-rich diet (Hope Farms, Woerden, the Netherlands) containing 2% cholesterol, 5% olive oil and 0.5% cholic acid. β-VLDL were isolated according to Redgrave (20) followed by a second identical centrifugation step.

Protein contents of the lipoprotein fractions were determined according to Lowry et al. (21). Total cholesterol, free cholesterol, triacylglycerols, and phospholipids were determined with enzymatic colorimetric assays (Boehringer Mannheim, Mannheim FRG, and Wako Chemicals GmbH, Neuss).

Labelling of lipoproteins

After isolation, the lipoproteins were immediately iodinated using the ^{125}I-iodine monochloride method described by Bilheimer et al. (22). After iodination the lipoproteins were dialysed and stored as described previously (8). The specific radio-activity ranged from 150 to 500 cpm/ng of protein.

Lipoprotein lipase

Bovine LPL was isolated from skimmed milk as described by Tajima et al. (23). Inactive LPL was obtained by incubation of the lipase for 4 hours at 50°C. Complete loss of activity of the enzyme was then checked using as substrate serum-activated [9,10-^{3}H] oleic acid-labelled trioleoyl-glycerol emulsified with phosphatidylcholine (24).

Binding studies

HepG2 cells, normal fibroblasts, and LDL receptor-negative fibroblasts were cultured in 2 cm^2 multiwell dishes (Costar) using Dulbbeco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) as previously described (8). LDL receptor-negative fibroblasts were obtained from a patient with homozygous Familial Hypercholesterolemia (25). Twenty-four hours before the start of the experiment, DMEM supplemented with 1% (w/v) human serum albumin (HSA) instead of FCS was added to the cells. The binding of ^{125}I-LDL and ^{125}I-VLDL to the cells in the presence or in the absence of LPL was determined after a 2.5 hour incubation with 10 μg/ml of ^{125}I-labelled lipoprotein at 0-4°C. After removing the medium the cells were washed five times with ice cold phosphate buffered saline (PBS) containing 1% (w/v) of bovine serum albumin (BSA), followed by one wash with PBS without BSA. Cells were then dissolved in 0.5 ml 0.2 N NaOH. Protein content was measured according to Lowry et al. (21). The radioactivity in an aliquot of the sample represents the binding.

To measure binding, intracellular-presence and degradation of lipoproteins separately, cells were incubated for 4 hours at 37°C with 10 μg ^{125}I-LDL or ^{125}I-VLDL per ml either in the presence or in the absence of LPL. At the end of the incubation the medium was removed

for determination of lipoprotein degradation as described previously (8). The cells were then washed five times with ice cold PBS/BSA (0.1%, w/v), followed by one wash with PBS without BSA. The cells were then released from the culture dishes by incubation with trypsin (0.05%, w/v) in a 137 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM D-Glucose, 0.02% EDTA buffer (pH 7.4) for 10 min at 37°C. The viability of the cells was checked, using trypan blue. Trypsin removes both cell-bound lipoproteins and cell-bound lipoprotein lipase (26). The cells were placed on ice to prevent further proteolysis, and then immediately centrifuged for 1 min at 13.000 g at 4°C. Radioactivity was determined in an aliquot of the supernatant, reflecting the binding of the labelled lipoproteins to the exterior of the cells. The cell pellet was resuspended in PBS and centrifuged for 5 min at 10.000 g. The pellet was dissolved in 0.5 ml 0.2 N NaOH. The radioactivity found in the pellet represents the amount of lipoprotein that is intracellularly present (trypsin-resistant). Protein was measured in an aliquot of the sample.

Treatment with heparinase (Sigma Chemical Company, St. Louis, MO, USA) was performed by incubating the cells at 37°C in the presence of 2.4 U/ml of heparinase.

Results

We have previously found that the LPL-mediated enhancement of the binding of LDL and VLDL occurs via bridging of LPL between heparan sulphate proteoglycans (HSPG) and lipoproteins, as it could be inhibited by pre-treatment of the HepG2 cells with heparinase (15). In Fig. 1 it is shown that preincubation of HepG2 cells with LPL for 1 hour at 4°C followed by washing, also results in an increase of the binding of LDL. This enhancement of the LDL-binding is similar to that found if the binding experiment is performed in the

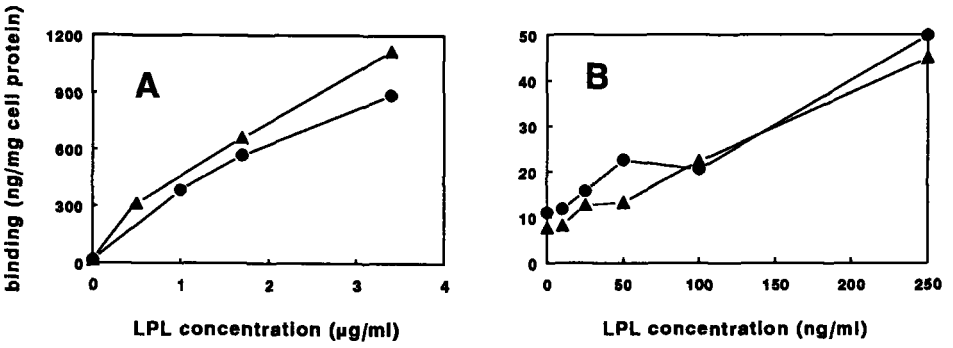

Figure 1. Effect of LPL on the binding of LDL. The binding experiment was performed either with HepG2 cells in medium containing increasing concentrations of LPL (▲) or with HepG2 cells that had been preincubated for a period of one hour with increasing concentrations of LPL at 0°C followed by washing in medium without LPL (●). Binding of ^{125}I-LDL was measured after 2.5 hours of incubation with 10 µg/ml of ^{125}I-LDL at 4°C, as described in Materials and Methods. Values are presented as the mean of three measurements. A: LPL concentrations in µg/ml range. B. LPL concentrations in ng/ml range.

presence of LPL. Therefore, these results indicate that the complex formation between the lipoproteins and the lipase prior to the binding is not a prerequisite, and thus sustain the hypothesis that LPL forms a bridge between HSPG and lipoproteins. In Fig. 1A LPL concentrations in the μg/ml range are used. In Fig. 1B it is shown that the LPL-mediated binding of LDL is already evident at more physiological concentrations of LPL (ng/ml range).

Figure 2 shows the binding of increasing concentrations of LDL to HepG2 cells, which had been preincubated with either medium alone or with medium supplemented with 1.7 μg/ml of LPL for a period of 1 hour at 0°C. The results show an approximately 20-fold higher maximum binding of LDL to the cells that had been preincubated with LPL. The Scatchard plots, shown in the insert of the graphs, suggest a comparable increase of the binding affinity.

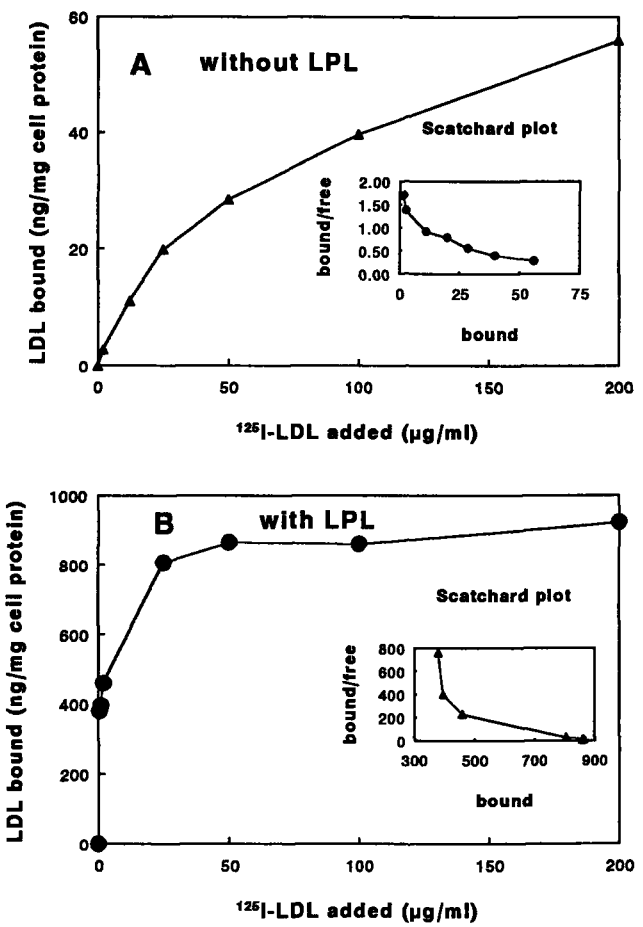

Figure 2. Binding curves of ^{125}I-LDL to HepG2 cells preincubated with DMEM/HSA alone (A) or with DMEM/HSA supplemented with 1.7 μg/ml of LPL (B). The cells were preincubated for a period of 1 hour at 4°C in the presence of DMEM/HSA alone or DMEM/HSA supplemented with LPL. After three washes with DMEM/HSA the cells were incubated with increasing concentrations of ^{125}I-LDL at 4°C for a period of 2.5 hours. Binding was then measured as the amount of ^{125}I-LDL that became cell associated as described in Materials and Methods. Values are the mean of two measurements. Inserts represent the respective Scatchard analysis.

To investigate whether LPL also enhances the uptake of LDL and VLDL, we incubated HepG2 cells with either ^{125}I-LDL or with ^{125}I-VLDL at 37°C either in the presence or in the absence of heat-inactivated LPL for a period of 4 hours. In Fig. 3 it is shown that, in the presence of heat-inactivated LPL, not only the binding of LDL and VLDL is enhanced (about 14-fold and 31-fold for LDL and VLDL, respectively) but also the internalization (expressed as the amount of intracellular plus degraded lipoprotein) is increased, although to a lesser extent (6-fold and 23-fold, for LDL and VLDL, respectively). In our previous paper (15) we have shown that the major part of LPL-mediated binding is prevented by pre-treating the cells with heparinase, indicating that the binding is mediated via HSPG. Figure 3 shows that, besides the inhibition of the LPL-mediated binding of LDL and VLDL, treatment of the cells with heparinase also resulted in an inhibition of the LPL-mediated internalization of both lipoproteins. These results indicate, therefore, that at least part of the lipoproteins which are bound via LPL to HSPG are subsequently internalized and degraded as well.

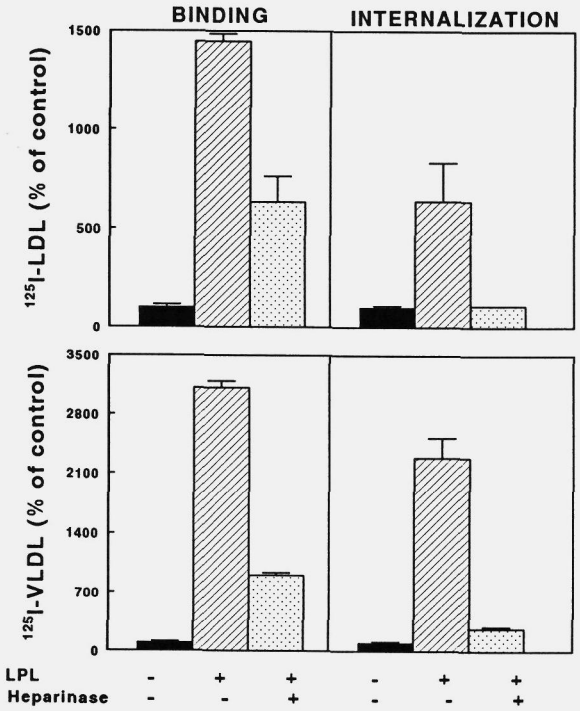

Figure 3. The effect of LPL and heparinase on the binding and internalization of ^{125}I-LDL and ^{125}I-VLDL by HepG2 cells. Lipoprotein binding and internalization (expressed as intracellular plus degraded lipoprotein) was measured upon incubation of the cells with 10 μg/ml of the labelled lipoproteins at 37°C for a period of 4 hours, in the absence (solid bars, control values 100%) or in the presence (tightly dotted bars) of 3.4 μg/ml of heat-inactivated LPL. For the heparinase treatment, 2.4 U/ml of heparinase were present during the 4 hours of incubation of the cells with labelled lipoprotein in order to prevent regeneration of HSPG on the cell membrane during this incubation period (dotted bars). Binding and internalization are expressed as a percentage of the control values (incubations in the absence of LPL), and were determined as described in Materials and Methods. Incubation with heparinase did not affect the control binding and internalization. The values represent the mean ± standard deviation of four measurements.

As shown in Table 1, degradation of both LDL and VLDL is inhibited in the presence of 50 μM chloroquine to 24% and 36% of the control value, respectively, when the experiment is performed in the absence of LPL, and to 32% and 38%, respectively, when performed in the presence of LPL. In the presence of 100 μM chloroquine the degradation of LDL and

Table 1. The effect of chloroquine and ammonium chloride on the degradation of ^{125}I-LDL and ^{125}I-VLDL, in the presence and in the absence of 3.4 μg/ml of heat-inactivated LPL.

	incubation with			
^{125}I-labelled lipoproteins	no addition	chloroquine 50 μM	chloroquine 100 μM	NH_4Cl 10 mM
	% of control degradation			
LDL	100	24 ± 5	10 ± 3	2 ± 0.3
LDL + LPL	100	32 ± 1	14 ± 1	2 ± 0.1
VLDL	100	36 ± 9	15 ± 1	10 ± 0
VLDL + LPL	100	38 ± 5	18 ± 2	6 ± 0.2

Twenty four hours before the start of the experiment, cells were incubated with DMEM/HSA (1% w/v). Degradation was determined after 4 hours of incubation of the cells with 10 μg/ml of ^{125}I-labelled lipoproteins in the presence or in the absence of LPL at 37°C with DMEM/HSA alone, or DMEM/HSA supplemented with chloroquine or ammoniumchloride as indicated. The degradation of the lipoproteins by the cells without any addition, and the degradation of the lipoproteins in the presence of LPL but without any further addition were taken as respective control values (100%). Values given represent the mean ± standard deviation of four measurements. The absolute control values were in ng lipoprotein degraded/mg cell protein: LDL, 140; LDL + LPL, 302; VLDL, 65; VLDL + LPL, 300.

VLDL is further reduced to 10% and 15% in the absence of LPL, and to 14% and 18% in the presence of LPL. 10 mM NH_4Cl reduces the degradation of LDL and VLDL to less than 10%, irrespective of the presence or the absence of LPL. From these results we conclude that the (LPL-mediated) HSPG-bound LDL and VLDL are also taken up and directed to the lysosomes for degradation.

We wondered whether or not lipoprotein receptors such as the LDL receptor and/or the putative remnant-receptor are involved in the internalization of LPL-mediated HSPG-bound LDL and VLDL. To answer this question, we first measured the binding and the internalization of ^{125}I-LDL and ^{125}I-VLDL in the presence and in the absence of LPL in normal fibroblasts and in LDL receptor-negative fibroblasts. Figure 4 shows that, in normal receptor-positive (*upper*) and receptor-negative cells (*lower*), the total amount of LDL and VLDL that is bound in the presence of LPL (hatched bars) is of the same order of magnitude. However, in contrast to the binding, the internalization of LDL and VLDL in receptor-negative fibroblasts did not reach the same order of magnitude as that measured for receptor-positive fibroblasts. Thus, although the LPL-mediated binding of LDL and VLDL occurs via HSPG, the major part of the subsequent internalization of these lipoproteins is mediated via the LDL receptor.

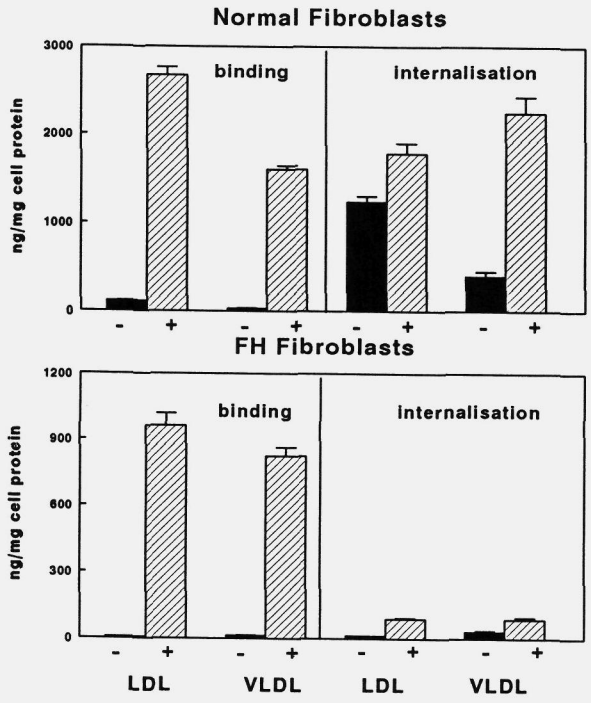

Figure 4. Effect of LPL on the binding and the internalization of LDL and VLDL by normal fibroblasts and LDL receptor-negative fibroblasts. The cells were incubated for 4 hours at 37°C, in the presence of 10 μg/ml of ^{125}I-LDL and ^{125}I-VLDL without (solid bars) or with (hatched bars) the addition of 3.4 μg/ml of LPL. The presence of LPL is also indicated by "+" and "-" signs in the figure. Binding and internalization are measured as described in Materials and Methods. The values represent the mean ± standard deviation of four measurements.

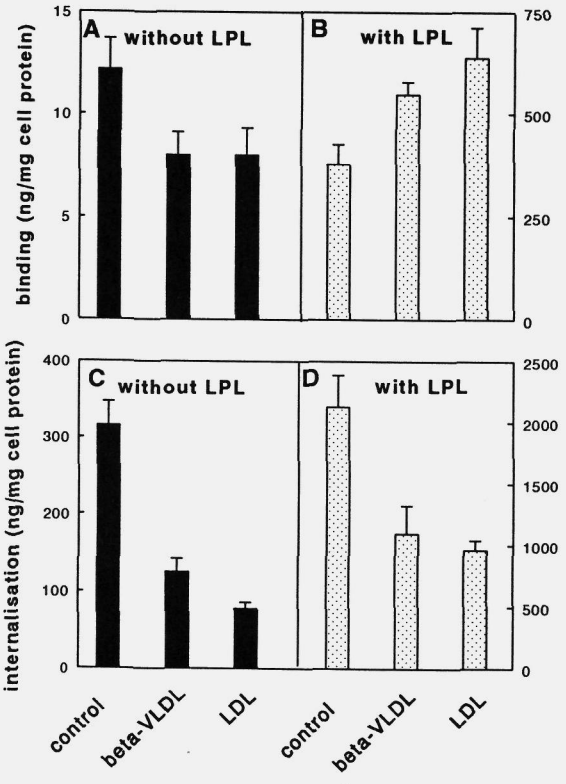

Figure 5. Effect of down-regulation of the LDL receptor on the binding and internalization of ^{125}I-LDL in the absence and in the presence of LPL. HepG2 cells were incubated in DMEM/HSA alone or DMEM/HSA supplemented with 200 μg/ml of rat β-VLDL or DMEM/HSA supplemented with 300 μg/ml of human LDL as indicated, at 37°C for a period of 24 hours. At the end of these incubations, the cells were washed three times with DMEM/HSA and incubated for 4 hours at 37°C with 10 μg/ml ^{125}I-LDL in the absence or in the presence of 3.4 μg/ml inactive LPL. Results are expressed as ng lipoprotein bound or internalized per mg cell protein. Values of binding and internalization in the absence of LPL (5A and 5C) are indicated on the left Y-axis, and values obtained after incubation in the presence of LPL (5B and 5D) are indicated on the right Y-axis.

Further evidence for this statement is provided by the results presented in Fig. 5. Pre-incubation of HepG2 cells with either 200 μg/ml of rat-β-VLDL or 300 μg/ml of human LDL resulted in down-regulation of the binding of ^{125}I-LDL to about 65% (Fig. 5A), whereas these conditions exerted an increase in the LPL-mediated binding to HSPG of about 1.5-fold (Fig. 5B). Pre-incubation of the cells with β-VLDL or LDL also resulted in a decreased internalization of LDL receptor-bound ^{125}I-LDL (40 and 25% of the control value, respectively) (Fig. 5C). Strikingly, parallel results were obtained for the uptake of LPL-mediated HSPG-bound LDL (50 and 45% of the control value, respectively) (Fig. 5D). From these results we conclude that the LDL receptor is involved in the uptake of lipoproteins following the binding of these lipoproteins via a LPL-mediated bridging between lipoproteins and HSPG.

We wondered whether the rate of internalization of HSPG-bound LDL is comparable to that of LDL receptor-bound LDL. To study this, the cells were first incubated with ^{125}I-LDL, either in the presence or in the absence of LPL, at 4°C for a period of 2 hours, followed by three washes at 4°C. To allow the lipoproteins to be internalized, the cells were then incubated at 37°C in medium, without any addition, for increasing periods of time. If expressed in absolute amounts of LDL internalized, it is obvious that the initial rate of internalization of LPL-mediated HSPG-bound LDL is comparable to the internalization rate of LDL receptor-bound LDL (0.58 versus 0.44 ng of LDL/minute/mg cell protein) (Fig. 6).

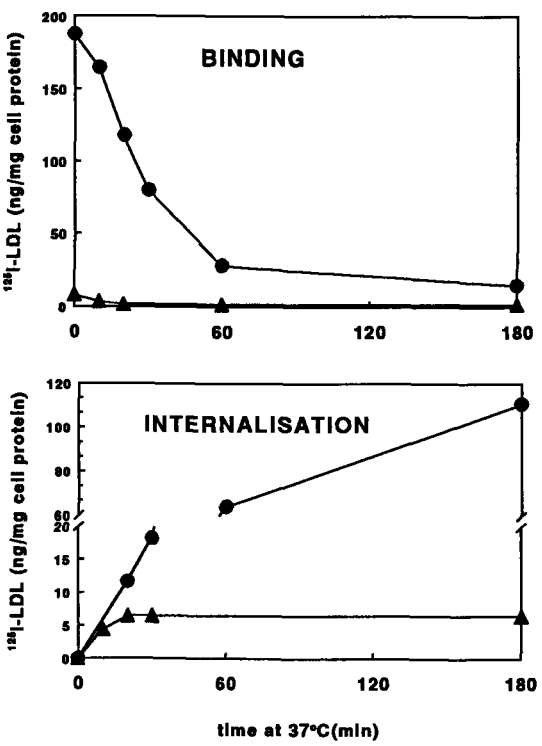

Figure 6. Rate of internalization of ^{125}I-LDL bound either in the absence or in the presence of 3.4 μg/ml of LPL. The HepG2 cells were incubated with 10 μg/ml of ^{125}I-LDL for 2 hours at 4°C either in the presence (●) or in the absence (▲) of 3.4 μg/ml of heat-inactivated LPL. At the end of this incubation period, the cells were washed three times with DMEM containing 1% (w/v) HSA. Cells were then further incubated at 37°C for increasing periods of time, as indicated, and the binding and internalization were measured separately as described in Materials and Methods. Values are expressed as ng ^{125}I-LDL per mg cell protein.

Discussion

Previously, it has been reported that LPL, independently of its lipolytic activity, enhances the cellular binding of a number of lipoproteins, including chylomicrons, VLDL, chylomicron- and VLDL-remnants, β-VLDL, apoE-free LDL and HDL (14,15,27). We found that neither the LDL receptor nor the LRP is involved in the LPL-mediated binding of LDL and VLDL, but that binding occurs mainly through bridging of LPL between HSPG on the plasma membrane and lipoproteins (15). This result was confirmed recently by Williams et al. (18). Further evidence for this is provided by the observation that preincubation of the cells with LPL followed by three washes resulted in the same increase in the binding of LDL as when the experiment was performed in the presence of the same amount of LPL (Fig. 1). The saturation curves shown in Fig. 2 indicate that the LPL-stimulated binding is due to an increase in the maximum binding and an increase in the binding affinity, of about 20-fold.

At 37°C most of the LPL-mediated binding and LPL-mediated internalization of LDL and VLDL could be inhibited by heparinase, indicating that the LPL-mediated increase in both binding and internalization are dependent on the presence of HSPG on the plasma membrane. Furthermore, our results show that the degradation of LDL and VLDL is lysosomal both in the absence and in the presence of LPL, which is similar to the results obtained by Williams et al. (18), with respect to LDL and Lp(a).

It has been suggested that the receptor involved in the LPL-mediated internalization of lipoproteins may be the LRP (14). We believe however that an important role for the LRP in this respect can be excluded, as the binding of apoE-free LDL (15) and Lp(a) (18) is also enhanced by the presence of LPL, while the LRP is assumed to bind only apoE-containing lipoproteins. In addition, as already mentioned by Williams et al. (18), most of the LPL-mediated binding is abolished by heparinase or heparitinase, whereas the LRP is assumed to contain no heparan sulphate side chains.

Bihain et al. (28) have found that long chain free fatty acids rapidly increase, up to 50-fold, the uptake of LDL. It seems, however, unlikely that a significant part of the LPL-mediated binding of LDL and VLDL is dependent on this "lipolysis stimulated receptor" as at 4°C LPL displays little or no activity at all and because heat-inactivated LPL has also been found to increase the binding to a similar extent as native LPL (15).

In their experiments, Rumsey et al. (19) found that also with receptor-negative fibroblasts the incubation with LPL resulted in a dramatic stimulation of the uptake of LDL, thereby stating that LPL increases lipoprotein uptake via a pathway not involving the LDL receptor. Although less pronounced due to a shorter incubation time (4 instead of 8 hours) and lower LPL concentration used (3.4 instead of 10 μg/ml), we also show that in LDL receptor-negative fibroblasts the internalization of LDL and VLDL is increased considerably upon incubation of the cells with LPL (Fig. 4). However, if expressed in absolute amounts of LDL taken up per mg of cell protein, our results show that the receptor-negative fibroblasts are much less efficient than control fibroblasts in LPL-mediated uptake of lipoproteins. This led

us to conclude that the major portion of the (LPL-mediated) HSPG-bound lipoproteins is taken up via the LDL receptor, whereas, simultaneously, only a minor part of the LPL-mediated binding of LDL is internalized without the action of the LDL receptor. Rumsey et al. (19) were not able to draw this conclusion as from their results a comparison of LPL-mediated uptake by receptor-negative fibroblasts with that of control fibroblasts could not be made.

We found that the LPL-mediated binding of lipoproteins is not suppressed by preincubation of the cells with lipoproteins (Fig. 5). This is in fully agreement with the results reported by Williams et al. (18) and Rumsey et al. (19). However, we also found that, in contrast to the binding, the uptake of HSPG-bound LDL is suppressed parallelly to the downregulation of the LDL receptor activity (Figs. 5C and 5D). This sustains our conclusion that the LDL receptor is indeed involved in the internalization of LPL-mediated HSPG-bound lipoproteins. Similar results were obtained for the degradation of HSPG-bound Lp(a) by Williams et al. (18). However, they found that down-regulation of the LDL receptor in normal fibroblasts did not affect the degradation of LDL in the presence of LPL, suggesting that the subsequent uptake of HSPG-bound LDL is LDL-receptor independent. An explanation for the discrepancy between results obtained by Williams et al. (18) and our results might be that they measured total cell association, thus without discriminating between lipoproteins bound to the outer cell membrane and those that had been internalized.

As shown in Fig. 6, most of the LDL receptor-bound LDL has been internalized within 10 to 15 minutes, which is in accordance with the recycling time reported earlier for the classical LDL receptor (28). These results also indicate that the internalization of HSPG-bound LDL, if expressed as ng of LDL/min/mg of cell protein, is as fast as the internalization of LDL bound to the classical LDL receptor (in the absence of LPL). Similar experiments have been performed by Rumsey et al. (19). However, in contrast to our conclusion, Rumsey et al. (19) concluded that the uptake of HSPG-bound LDL is much slower than the uptake of LDL that is bound directly to the LDL receptor. Two facts may explain the discripancy between their conclusion and our conclusion regarding the rate of uptake of HSPG-bound LDL: (i) they used one hour as first sampling time point, whereas we used minutes for initial time intervals, which is in our opinion reasonable as the LDL receptor recycling time is also in the order of minutes (28); (ii) in their time course experiment, Rumsey et al. (19) expressed the rate of internalization as "% of total radioactivity". We also found much slower internalization when expressed as "% of total radioactivity". However our results clearly show that the internalization of LPL-mediated HSPG-bound LDL is equally fast as that of LDL receptor-bound LDL, when expressed in absolute terms ("ng of LDL/min/mg of cell protein").

From the results presented in this paper, we propose the mechanism for LPL-mediated uptake of LDL and VLDL as illustrated in Fig. 7: LPL enhances the binding of LDL and VLDL to cells by means of a bridging between the lipoproteins in the medium and HSPG on the plasma membrane. Thereafter, the HSPG-bound LDL and VLDL are internalized mainly via the rapid process of classical LDL receptor recycling system, if the LDL receptor

is present. Simultaneously, the remaining portion of HSPG-bound lipoproteins is internalized together with HSPG, which is a much slower process with a half-life of about 7 hours (30). In LDL receptor-negative fibroblast the total amount of HSPG-bound lipoprotein is internalized via this slow process of HSPG uptake. The fact that in normal cells the uptake of LPL-mediated HSPG-bound LDL continues up to 60 minutes indicates that the LDL-receptor recycling system is saturated during 4 to 6 LDL receptor cycles and, consequently, the rate limiting step in this process.

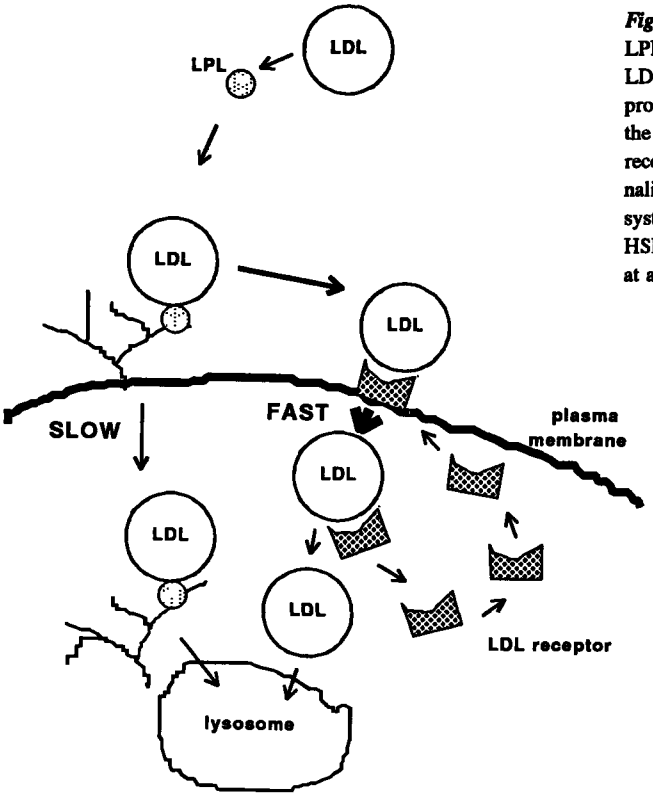

Figure 7. **Proposed mechanism for LPL-mediated binding and uptake of LDL. After binding to heparan sulphate proteoglycans (HSPG), the major part of the LDL is transferred to the LDL receptor, whereafter it is rapidly internalized via the LDL receptor recycling system. Only a minor portion of the HSPG-bound LDL is taken up directly, at a much slower rate.**

The role of LPL in lipoprotein uptake *in vivo* is presently the subject of speculation. Williams at al. (18) suggest that LPL may serve as an atherogenic molecule in the arterial wall, by stimulating the uptake of apoB-rich lipoproteins by macrophages and smooth muscle cells, leading to foam cell formation. On the other hand, in the liver it would be anti-atherogenic by enhancing uptake of apoB-rich atherogenic lipoproteins, such as VLDL-remnants, LDL and Lp(a). This possible dual function of LPL *in vivo* may thus relate to its location.

It has been reported that macrophages in atherosclerotic plaques synthesize LPL, which can be found anchored to their cell surface (31,32). This fact, together with the observation that *in vivo* macrophages synthesize HSPG depending on the amount of intracellular

cholesterol-ester accumulation (30), strongly sustains the hypothesis that in the arterial wall LPL may, indeed, serve as an atherogenic factor.

The concentration of LPL in the circulation is normally kept low because of avid uptake in the liver (33). Although the lipase concentrations used in most of the present experiments are more than 100 times the physiological concentration that ranges between 8 and 25 ng/ml (34), we found that in the presence of 25 ng/ml of LPL the binding of LDL to HepG2 cells also increased (about 1.5-fold, Fig. 1B). This sustains the hypothesis that *in vivo* LPL may affect lipoprotein binding. Whether this may also affect the lipoprotein catabolism *in vivo* remains subject to further investigation.

In vivo, most of the circulating LPL is associated with lipoproteins (34), mainly with LDL and HDL and, strikingly, not with VLDL or chylomicrons (35), suggesting a specific role for LPL in directing LDL and HDL to the liver. Vilaro et al. (36) have shown that exogenous LPL bound in the liver caused a dramatic increase in the utilization of a perfused triacylglycerol emulsion. Possibly, LPL fulfils a metabolic role at its binding-site in the liver before it is degraded. Hepatic lipase which is present in the liver could also act in this way (37).

The relevance of our data, obtained with cells in culture, for the *in vivo* fate of lipoproteins is still unclear. We propose that LPL may accelerate the removal of the atherogenic LDL particles from the blood circulation and, simultaneously, stimulate the reverse cholesterol transport mediated by HDL. Studies are in progress to test these potential important implications for atherosclerosis.

Acknowledgements

This study is financially supported by the Netherlands Heart Foundation (MM: project #87.025; PL: project #89.057). We are grateful to Leny de Pagter for cultivating the cells.

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Chapter 7

AN ACCEPTOR SPLICE SITE MUTATION IN INTRON 16 OF THE LOW DENSITY LIPOPROTEIN RECEPTOR GENE LEADS TO AN ELONGATED, INTERNALIZATION DEFECTIVE RECEPTOR

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Atherosclerosis, in press.

Summary

In this report, we describe the characterization of a mutation in the low density lipoprotein (LDL) receptor gene of a true homozygous familial hypercholesterolemic (FH) patient. The combined use of denaturing gradient gel electrophoresis (DGGE) and DNA sequence analysis revealed a unique A to G transition in the penultimate 3'-nucleotide of intron 16 of the LDL receptor gene, which disrupts the acceptor splice site. cDNA sequence analysis indicated that a cryptic splice site was activated in intron 16, upstream from the original splice site, leading to the inclusion of 62 nucleotides and a reading frame-shift. The resulting new translation product contains a stretch of 154 amino acids at the carboxy-terminal that have no resemblance to the normal receptor protein. To elucidate the biological effects of the mutation, the structural and functional properties of the mutated LDL receptor protein were studied. Immunoprecipitation of the newly synthesized LDL receptors showed that an aberrant precursor form of the LDL receptor protein was synthesized, about 10 kD larger than normal, which is not further processed to the mature form. Some 50% of the normal LDL binding activity was found on the cell surface of the patient's fibroblasts, whereas internalization and degradation of LDL were abolished.

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominantly-inherited disease caused by mutations in the gene for the low density lipoprotein (LDL) receptor, which mediates the specific uptake of plasma LDL. In most populations, the heterozygous form of FH is rather common, affecting approximately one person in every five hundred. Heterozygous FH individuals have only half the normal number of functional LDL receptors and, as a consequence, plasma LDL cholesterol levels of about twice the normal level, leading to an increased risk of premature atherosclerosis. Homozygous FH patients are more severely affected and rarely reach the age of maturity [1].

After the LDL receptor gene was cloned [2], a vast array of mutations including insertions, deletions, nonsense and missense mutations have been described, affecting either the synthesis, post-translational processing, ligand binding activity or the internalization of the LDL receptor [3]. So far, most mutations characterized appeared to be unique, with the exception of those cases, in some confined populations, where a founder gene effect may exist [4-8].

The characterization of additional mutations not only provides further insights in the relationship between the structure and function of the LDL receptor protein, but also allows for an accurate diagnosis on which treatment and counselling can be based. In addition, since it has been reported that certain LDL receptor gene mutations may play a role in the efficacy of the therapeutical treatment, comparison between groups of patients with different

mutations might lead to an optimal therapy [9].

In this report we describe a LDL receptor mutation in a true homozygous FH patient. The underlying molecular defect consists of an A to G transition in the penultimate 3'-nucleotide of intron 16, which disrupts the acceptor splice site. The activation of an upstream cryptic splice site in intron 16 caused the inclusion of 62 nucleotides, leading to a reading frame-shift and, consequently, to an elongation of the receptor protein by 90 amino acids.

Materials and Methods

Subjects

MA, a 25-year-old woman, was given a clinical diagnosis of homozygous familial hypercholesterolemia at the age of two, based on markedly-increased concentration of plasma cholesterol (ranging from 17.9 to 29.3 mmol/l) and the presence of typical tendon xanthomas. Her parents had elevated serum cholesterol levels. They were not consanguineous, within at least two generations. Four members of the father's family died of presumed myocardial infarction between 54 and 64 years of age.

At the age of 13 the patient underwent a portacaval shunt operation which resulted in a strong reduction of the plasma cholesterol level down to 12.8 mmol/l and in a dramatic regression of the xanthomas.

DNA isolation and analysis

Genomic DNA was isolated from peripheral leukocytes according to the method of Miller [10]. Restriction fragment length polymorphism (RFLP) haplotype analysis and Southern blot analysis of the LDL receptor gene were performed as previously described [8,11].

To screen for small deletions, insertions or point mutations in the LDL receptor gene, denaturing gradient gel electrophoresis (DGGE) was applied. Briefly, genomic DNA was amplified using intron-specific primers for each of the 18 exons encompassing the coding and the splice site consensus sequences at the intron/exon junctions. PCR primers were designed on the basis of the sequence published by Leitersdorf et al. [6] and synthesized on a Bioresearch Cyclone™ DNA synthesizer. One primer of each set contained a GC-sequence of 15 nucleotides. This GC-rich sequence was further elongated to a 50 nucleotides GC-clamp in a second PCR run, thereby providing an artificial high melting domain in the DGGE analysis [12]. As an example, PCR of exon 17 was carried out on approximately 500 ng of genomic DNA in a buffer containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxy nucleoside 5'-triphosphate (dNTPs), 50 pmol each of the primers 2768 (5'-CGCCGCCGCCGCCCGTGACAGAGCGTGCCTC-3') and 2769 (5'-TGGCTTTCTAGAGAGGGTC-3') and 1 unit of *Taq* DNA polymerase (Amplitaq, Cetus Perkin Elmer, Norwalk, Conn.) in a final volume of 50 μl. The amplification protocol consisted of 32 cycles of 1 minute denaturation at 94°C, 30 seconds annealing at 55°C and

90 seconds extension at 72°C. For amplification of the other 17 exons, similar conditions were used with minor variations. The experimental details of the amplification protocol of each exon will be reported elsewhere (Lombardi, P. et al., manuscript in preparation). The abundance and size of the PCR products were analyzed by electrophoresis on 2% agarose gel.

DGGE was carried out according to Fischer and Lerman [13]. All samples were run on 9% polyacrylamide gels containing a 40-80% linearly-increasing denaturing gradient (100% denaturant is equivalent to 7 M urea and 40% (v/v) deionized formamide). Gels were run for at least 20 hours at 60°C at 80 Volt (6 Volt.cm^{-1}) in 40 mM Tris-acetate, 1 mM EDTA, pH 7.5 (TAE) buffer. When DGGE analysis of artificially formed heteroduplexes was performed, PCR products from two different subjects were mixed and heat-denaturated at 95°C for 5 minutes, after which the DNA solution was slowly cooled to room temperature. After electrophoresis, gels were stained in ethidium bromide and examined under UV illumination.

For sequencing, the PCR fragment containing the mutation was ligated into the pT7 blue vector (pT7 blue cloning kit, Novagen, Madison, WI, U.S.A.) according to the manufacturer's recommendations. Double-stranded sequencing was performed using the ^{T7}Sequencing kit (Pharmacia, Uppsala, Sweden) with ^{35}S-αdATP (10 mCi/ml, Amersham), according to the protocol supplied by the manufacturer.

mRNA isolation and analysis

Total cytoplasmic RNA was isolated from fibroblasts that had been incubated for 12 to 16 hours with medium containing lipoprotein-deficient serum (LPDS), according to the method of Chomczynsky and Sacchi [14]. Poly(A)-RNA was isolated from total RNA by oligo-dT cellulose affinity chromatography (Pharmacia, Uppsala, Sweden) [15].

Northern blotting was carried out according to standard procedures [15] using the LDR-receptor probe 2HH1 [8].

cDNA synthesis and analysis

cDNA was synthesized by using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, U.S.A.), according to the manufacturer's recommendations. Each reaction mixture contained 4 μg of total RNA and 50 pmoles of primer 1834 (5'-CACTGAACAAATACAGCAAC-3'), located at position 4835-4855 of the LDL receptor gene. Five μl of the resulting cDNA were used as a template for PCR amplification with primers 295 (5'-TCTGGGCGACGTTGCTGGCA-3'), located at position 2313-2233 (exon 16) and SP11 (5'-GCTTTGGTCTTCTCTGTCTTTGAAT-3'), located at position 2645-2670 (exon 18) of the LDL receptor gene [6]. PCR was carried out in a buffer containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each primer and 2 units of *Taq* DNA polymerase (Amplitaq) in a final volume of 50 μl. The amplification protocol consisted of 45 cycles of 1 minute denaturation at 94°C, 1 minute and 30 seconds annealing at 60°C and 2 minutes extension at 72°C. The amplified fragments

were analyzed by electrophoresis on 2% agarose gel and by Southern blotting using probe 2HH1 [8]. The products were cloned and sequenced as described above.

Cell culturing

Fibroblasts from explants obtained by skin biopsy of the proband, her mother and a control subject were maintained in culture in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml).

One week prior to the experiments, cells were seeded in 2 cm^2 multiwell dishes (Costar) or in 100-mm dishes. Twenty-four hours before the assays, the cells were washed with Puck's saline (0.136 M NaCl, 5.3 mM KCl, 4 mM $NaHCO_3$, 0.1% (w/v) glucose, 5 mg/l Phenol red, pH 7.4) and further incubated with DMEM containing 10% (v/v) LPDS instead of FCS, to stimulate the LDL receptor expression.

Synthesis and processing of the LDL receptor protein

Immunoprecipitation of ^{35}S-labelled LDL receptor protein from cultured cells was performed essentially as described previously [16]. Cells in 100-mm dishes were washed twice with Puck's saline and incubated for 2 hours at 37°C in 5 ml of methionine-free medium (DMEM without methionine) containing 5% LPDS (2.5 mg protein/ml) and 0.1 μM ^{35}S-methionine (20 μCi/ml, specific radioactivity 200 Ci/mmole) (Amersham, Buckinghamshire, U.K.). In pulse-chase experiments, the medium containing ^{35}S-methionine was removed after the pulse-period and replaced with 10 ml of DMEM containing 200 μM methionine and 5% (v/v) LPDS, and the incubations were continued for another 2 hours. At the end of the incubation period the cells were washed twice with ice-cold PBS and solubilized with 350 μl Triton X100-containing buffer per 100-mm dish [17]. Extracts from three identical dishes were pooled and centrifuged at 100,000 x g for 30 minutes at 4°C. To measure incorporation of radioactivity into LDL receptor protein, the bulk of Triton-soluble cell extract was immunoprecipitated with preformed immunocomplexes of monoclonal antibody IgG-C7 (anti-LDL receptor) [18] or IgG-1-3-1 (anti-tissue-type plasminogen activator) (t-PA) [19] with goat anti-mouse IgG [17]. The immunoprecipitates were washed and solubilized, and the radioactive proteins separated by electrophoresis and detected by autoradiography.

Binding assays

LDL were isolated and iodinated with ^{125}I as described before [20].

The ability of cells to bind, internalize and degrade ^{125}I-LDL at 37°C was measured at the indicated ^{125}I-LDL concentrations as described previously [21]. After incubation with ^{125}I-LDL in DMEM/1% HSA at 37°C for 3 hours, cells were rapidly cooled to 4°C. The medium was immediately harvested and its content of non-iodide trichloroacetic acid soluble radioactivity was measured (LDL degradation). The cells were washed four times with phosphate buffered saline (PBS) containing 0.2% albumin and two times with PBS alone. Thereafter the cells were incubated with 0.05% (w/v) trypsin, 0.02% EDTA (w/v) in PBS

for 10 minutes at room temperature. The trypsin-releasable radioactivity was taken as the surface-bound fraction. Subsequently, the cells were solubilized in 1 M NaOH and the radioactivity was counted (LDL internalization). An aliquot of the cell lysate was used for protein determination [22]. Values for the specific (receptor-mediated) binding, internalization and degradation were calculated by subtracting the amount of labelled LDL that was bound, internalized or degraded in the presence of a 30-fold excess of unlabelled LDL (non-specific binding) from the amount of labelled LDL that was bound, internalized or degraded in the absence of excess of unlabelled LDL (total binding).

Results

Family analysis

The pedigree of the family of the proband is shown in Fig. 1. The plasma lipid values are given in Table 1. The maternal grandfather died at the age of 92. The maternal grandmother, who was reported to have Achilles tendon xanthomas, died at the age of 85. The paternal grandfather was 57 years old when he died of heart disease and 3 of his siblings also died of heart diseases between the ages of 54 and 64 years. All the family members reported in Table 1 were diagnosed as having FH. No other cases of coronary heart diseases were recorded in the family. Tendon xanthomas were present in all subjects whose lipid parameters were determined, with the exception of subjects 3 and 4 in the third generation. The proband is 25 years old; she has a 6-year-old daughter and she is reported to be well. MA is currently treated with 1000 mg probucol, 80 mg aspirin and 100 mg atenolol.

Table 1. Biochemical and clinical data of MA's family.

Generation	Subject	Age	Chol*	TG*	LDL-Chol*	HDL-Chol*	CAD‡	Xant.‡
II	2	54	8.56	0.64	6.46	1.49	—	+
	3	65	10.00	1.12	7.65	1.49	—	+
	4	64	9.69	3.45	6.63	1.16	—	+
	5	45	10.62	1.13	10.24	0.67	—	+
III	1§	13	21.9	0.9	20.66	0.85	+	+
	1‖	25	11.68	0.7	9.29	1.19		
	2	28	9.16	1.12	7.52	1.10	—	+
	3	36	11.11	1.72	8.60	1.10	—	n.a.¶
	4	30	8.12	3.24	5.61	1.20	—	n.a.¶

* in nmol/l.

‡ — and + represent the absence or presence of coronary artery disease and xanthomas, respectively.

§ Before and ‖ after portacaval shunt operation.

¶ n.a. indicates data not available.

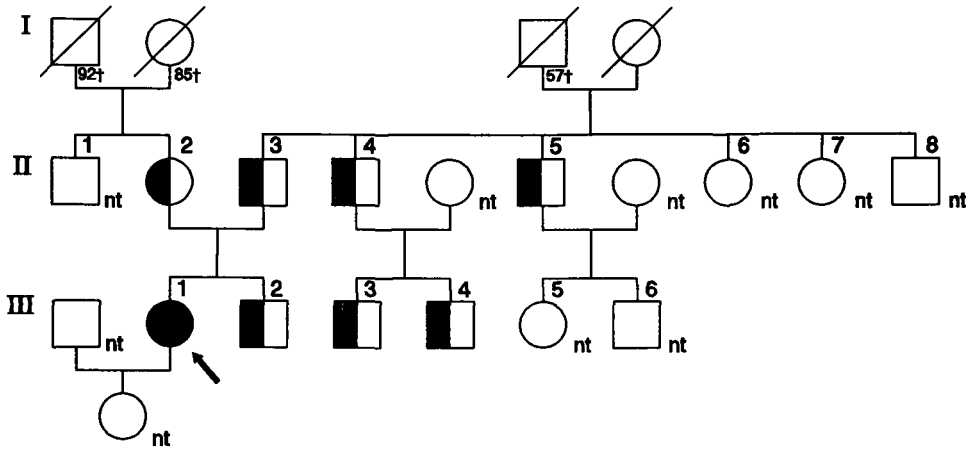

Figure 1. MA pedigree. The homozygous proband is indicated by an arrow. Those members of the family known to have an elevated cholesterol concentration, consistent with a diagnosis of heterozygous FH, are indicated by half-filled symbols. The open symbols represent family members whose lipid parameters have not been tested (n.t.).

Southern and Northern blotting

Southern blot analysis after *Bgl*II digestion of genomic DNA isolated from the proband, her parents and her brother did not reveal any major abnormality in the LDL receptor gene (not shown).

Restriction fragment length polymorphism (RFLP) analysis was carried out on DNA isolated from MA, her mother and her father. MA was homozygous at each of the polymorphic sites scored (*Bsm*I, *Sph*I, *Stu*I, *Ava*II, *Spe*I, *Apa*LI-3', *Apa*LI-5', *Pvu*II, *Nco*I). This suggests that she had inherited the same mutant allele from both parents (not shown). The same haplotype was found with a frequency of 13.5% in individuals of Caucasian American descendant as described by Leitersdorf et al. [11].

Northern blot analysis revealed that the LDL receptor mRNA of MA and her mother did not significantly differ in size (5.3 kb) from the mRNA obtained from HepG2 cells and from fibroblasts of a normal individual (not shown). Hybridization of the same blot with a control (actin) probe showed that also the relative abundance of MA's mRNA was similar to that of normal fibroblasts.

Identification of a sequence alteration by DGGE

The absence of any gross abnormality in the LDL receptor genes of the proband suggested the presence of a point mutation or a minor rearrangement. To determine the exact location of the mutation, the amplified products of all 18 exons, including the splice site sequences, of the LDL receptor gene of MA were first subjected to DGGE analysis [13]. As shown in Fig. 2, a unique mutation was found to be located in exon 17 or in its splice junctions. The

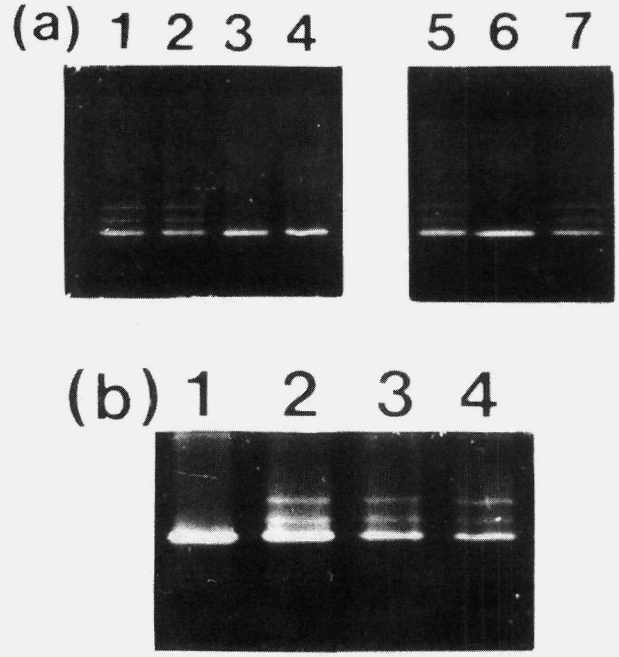

Figure 2. DGGE analysis. Exon 17 was amplified from genomic DNA as described in Materials and Methods. The PCR product, containing a 50-bp GC clamp was loaded on a 9% polyacrylamide gel containing a 40-80% denaturing gradient (100% denaturant is equivalent to 7 M urea and 40% (v/v) deionized formamide). The ethidium bromide staining is shown. (A) Lane 1: mother; lane 2: father; lane 3: MA; lane 4: normal subject; lane 5: heteroduplexes formed with the PCR obtained from MA and a normal individual, mixed in equal proportions, boiled for two minutes and slowly cooled down to room temperature; lane 6: PCR products of two normal individuals were treated as in lane 5; lane 7: MA's brother. (B) Lane 1: MA; lane 2: heteroduplexes formed with the PCR obtained from MA's mother and father, mixed in equal proportions, boiled for two minutes and slowly cooled down to room temperature; lane 3: PCR products of MA and her father were treated as in lane 2; lane 4: PCR products of MA and her mother were treated as in lane 2.

appearance of heteroduplex bands in the DGGE pattern of MA's parents and brother (Fig. 2A, lanes 1, 2 and 7) clearly indicated the presence of a mutation, although in MA the DGGE pattern was not altered (Fig. 2A, lane 3). However from the DGGE pattern of both parents and brother it was apparent that both homoduplex bands, corresponding to the mutant and normal allele respectively, migrate at the same position. To verify the hypothesis that the melting temperature of the mutant homoduplex band did not differ from that of the normal homoduplex band, the PCR product of exon 17 of MA was mixed with the one of a normal subject by denaturation at 94°C and reannealing at room temperature. In case of a homozygous mutation in MA, artificially-formed heteroduplex bands will appear after mixing the PCR products. Indeed, the electrophoretic pattern produced was the same as obtained with MA's heterozygous relatives (Fig. 2A, lane 5), while the mixture of two normal subjects produced the original single band (Fig. 2A, lane 6).

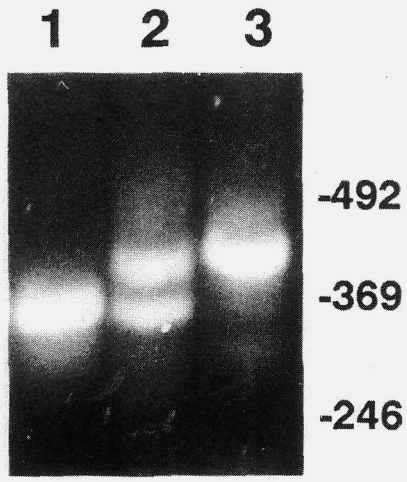

Figure 3. RT-PCR. Total mRNA from HepG2 cells, MA and MA's mother fibroblasts was used for reverse transcription. The resulting product was amplified by PCR using primers 295/SP11 as described in Materials and Methods. Samples were run in a 2% agarose gel. The ethidium bromide staining of the gel is shown. Lane 1: HepG2 cells; lane 2: MA's mother; lane 3: MA. The position of the size markers (in base pairs) is indicated.

Identical banding patterns for both parents (Fig. 2A, lanes 1 and 2) strongly indicated that they carry the same mutant allele. This was further confirmed by DGGE analysis of the mixture, followed by heat-denaturation and reannealing, of the PCR products of MA's mother and father and of MA with each parent (Fig. 2B, lanes 2, 3 and 4). Since no additional bands are visible, we conclude that MA has inherited the same mutation from both parents.

Nucleotide sequence of the mutant

The amplified product including exon 17 and splice site sequences at intron-exon junction of MA was cloned. Sequence analysis revealed an A to G transition in the penultimate 3'-nucleotide of intron 16, thus altering the 3'-splice acceptor AG dinucleotide to GG (not shown).

cDNA synthesis and sequence analysis

To investigate the effect of this splice site mutation on the processing of the LDL receptor mRNA, cDNA was synthesized from total RNA isolated from fibroblasts of both MA and her mother. mRNA extracted from HepG2 cells was used as a control. The reverse transcriptase product was subjected to PCR amplification with primers in exons 16 and 18 (see Materials and Methods) and analyzed using a 2% agarose gel. As shown in Fig. 3, MA cDNA appears as a single band of about 440 bp (lane 3), about 70 bp larger than the cDNA produced by HepG2 cells (lane 1), which has the expected size of about 370 bp. cDNA from the mother (lane 2) shows two products, corresponding to the fragment of expected size (370 bp) and to the 440 bp cDNA, respectively. Both products hybridized with a LDL receptor cDNA probe [8] (not shown).

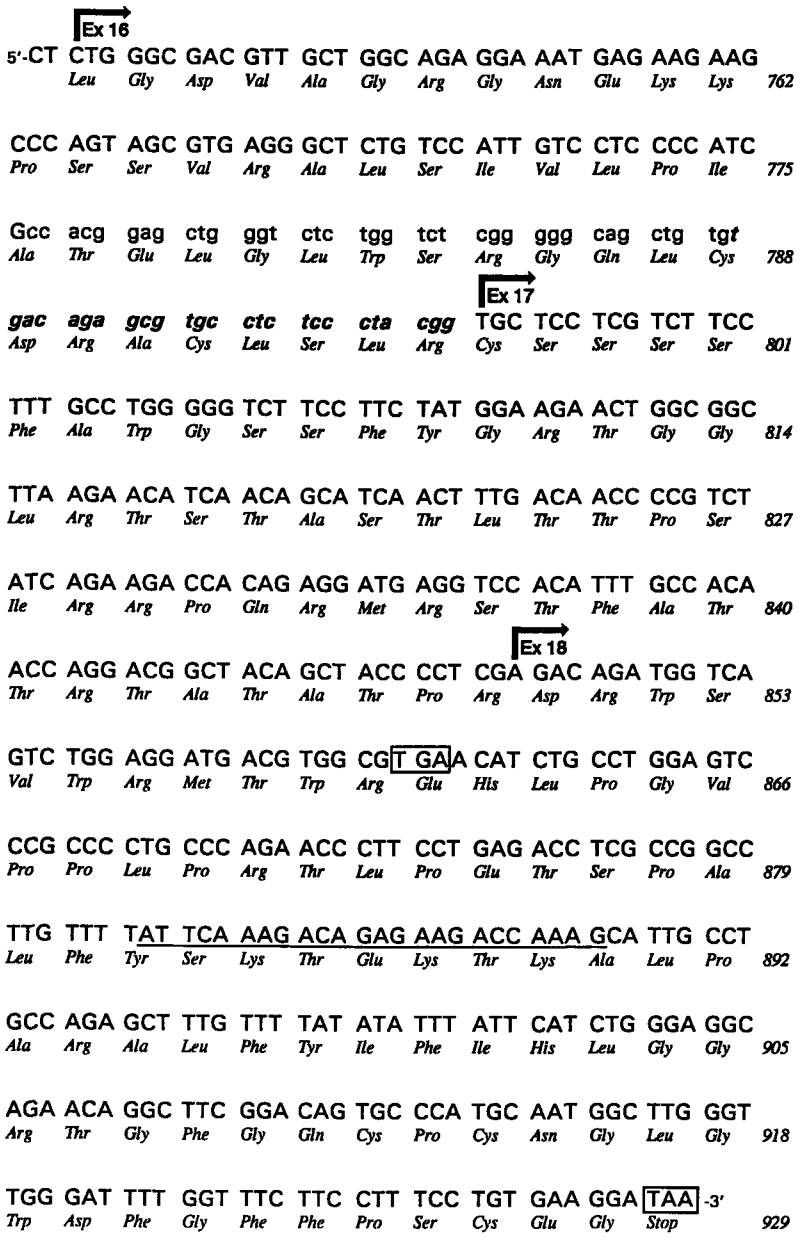

Figure 4A. Nucleotide sequence of the cDNA of the mutant LDL receptor gene and the predicted amino acid sequence of the protein. The first nucleotides of exons 16, 17 and 18 in the normal sequence are indicated with an arrow. The lower cases indicate the 62 nucleotide inclusion. The homology with the known sequence of intron 16 is marked in bold. The underline represents primer SP11, located in exon 18. The two stop-codons, TGA in the normal sequence and TAA in the mutant are boxed. The amino acids are numbered on the right-hand side.

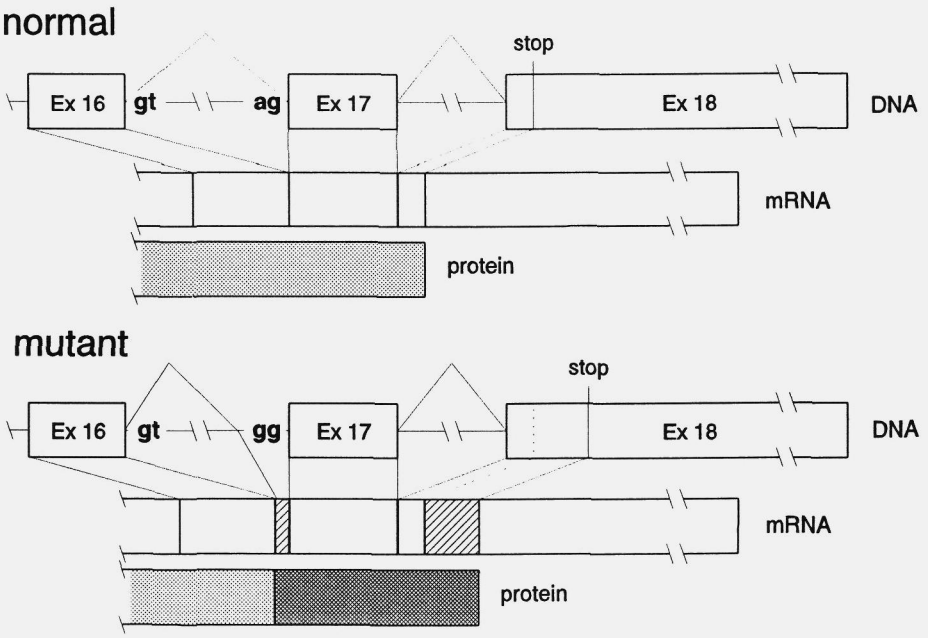

Figure 4B. Splicing of the normal (upper) and mutant (lower) mRNA. The boxes represent exons; the horizontal lines represent introns. The mutation A to G in the highly conserved AG sequence is indicated. The hatched boxes represent the 62 bases inclusion between exon 16 and exon 17 and the elongation of 204 bases in exon 18, respectively. The light shaded boxes represent normal protein sequence; the dark shaded box represents protein sequence produced by the mutation. The drawing is not to scale.

Sequence analysis of the 440 bp fragment revealed a 62 nucleotide insert between exon 16 and exon 17 (Fig. 4A,B). The last 25 nucleotides of this fragment were identical to the known sequence of the 3'-end of intron 16 [6], with the exception of the penultimate nucleotide which is a G to A substitution, as could be anticipated from the sequence analysis of the splice site consensus sequence at the intron 16/exon 17 junction. The homology with the 3'-end sequence of intron 16 suggests that the insertion is due to the activation of a cryptic splice site in intron 16. This inclusion causes a frame-shift, leading to a new in-frame stop-codon (TAA) in exon 18, 204 nucleotides downstream of the TGA stop-codon present in the normal sequence (Fig. 4A).

Synthesis and processing of the LDL receptor protein

To study the biosynthetic pathway and processing of the mutant LDL receptor protein, fibroblasts of MA, her mother and a normal subject were pulse-labelled with ^{35}S-methionine for 2 hours. Thereafter, the LDL receptor protein was immunoprecipitated from the cell extracts with monoclonal anti-LDL receptor antibody IgG-C7 immediately (Fig. 5, lanes 2, 5 and 8) or, after a subsequent 2 hours chase period, with unlabelled methionine (Fig. 5,

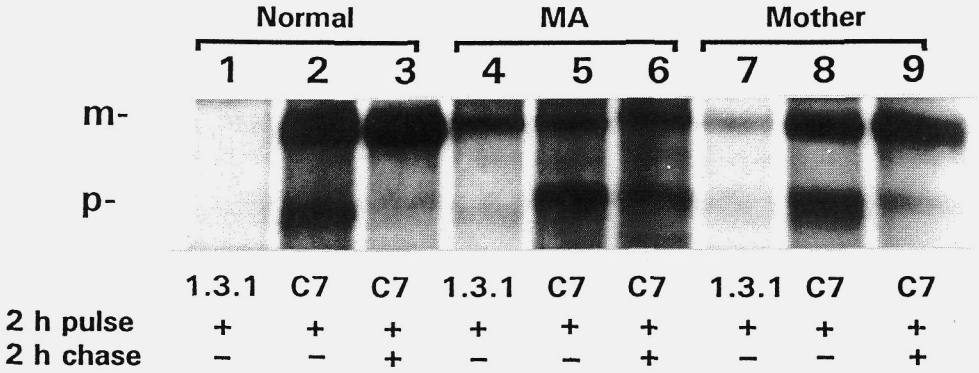

Figure 5. Immunoprecipitation of ^{35}S-methionine-labelled proteins. Fibroblasts were pulse-labelled with ^{35}S-methionine for 2 hours (lanes 1, 2, 4, 5, 7, 8) and subsequently chased for 2 hours with unlabelled methionine (lanes 3, 6, 9). Cell extracts were incubated with preformed immunocomplexes containing IgG 1-3-1 (anti-t-PA) monoclonal antibody (lanes 1, 4, 7) or IgG-C7 (anti-LDL-receptor) monoclonal antibody (lanes 2, 3, 5, 6, 8, 9). The immunoprecipitates were subjected to SDS gel electrophoresis and autoradiography. The position of the mature (*m*) and precursor (*p*) forms of the receptor protein are indicated.

lanes 3, 6 and 9). To correct for the presence of non-specific immunoprecipitation products, cell extracts were also immunoprecipitated with a monoclonal antibody directed against an irrelevant antigen (anti-t-PA, Fig. 5, lanes 1, 4 and 7). After a 2-hours pulse, MA cells synthesized a receptor protein with an apparent molecular weight of 131 kD (lane 5), which is larger than the precursor form and smaller than the mature form of the normal LDL receptor (120 and 160 kD, respectively) (lane 2). A band of the same molecular weight was still present after 2 hours of chase (lane 6), suggesting that this protein is not further processed. Fibroblasts from MA's mother produced, after 2 hours pulse, both the precursor and mature form of the normal LDL receptor and the aberrant 131 kD protein (lane 8), which was still present after 2 hours of chase (lane 9). A band having the same apparent molecular weight as the mature LDL receptor protein appears in the immunoprecipitate of MA cells (lanes 5, 6). However, the same band is also present in the anti-t-PA-immunoprecipitate of both MA (lane 4) and her mother's fibroblasts (lane 7), suggesting that this band represents a non-specific product. These results indicate that a LDL receptor protein with an increased molecular weight is synthesized by MA cells. Since no specific bands smaller than the one at 131 kD were visible in the immunoprecipitate of MA cells, we postulate that this band represents the precursor form of the LDL receptor protein.

Binding studies

To assess the functional properties of the mutated LDL receptors, fibroblasts from skin biopsies were cultured and their ability to bind, internalize and degrade ^{125}I-LDL was

investigated. As Fig. 6 shows, MA cells retain about 50% of functional LDL receptors on the cell surface as compared with control fibroblasts (binding, panel A), while no detectable internalization or degradation was found in MA cells, even at the fairly high concentrations of LDL used (panel B and C).

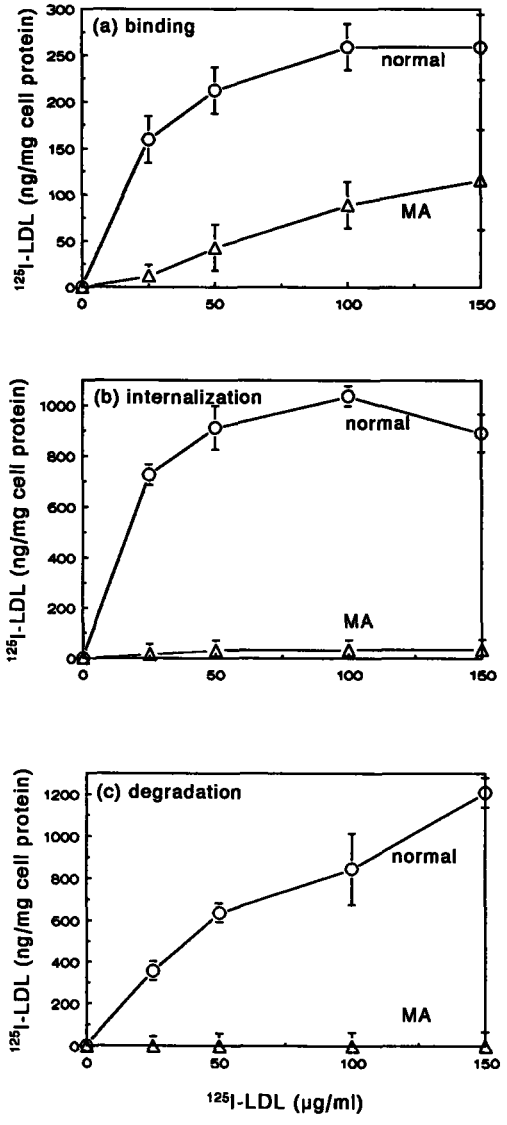

Figure 6. LDL receptor mediated binding, internalization and degradation of ^{125}I-LDL to fibroblasts from MA (△) and a normal subject (○). After 24 hours incubation in 10% LPDS, fibroblasts were incubated for 3 hours at 37°C in medium containing 1% HSA and the indicated amounts of ^{125}I-LDL (spec. act. 150 cpm/ng) in the absence or presence of a 30-fold excess of unlabelled LDL. Surface binding (A), internalization (B) and degradation (C) were measured as described in Materials and Methods. Each value represents the mean of triplicate incubations ± S.D.

Discussion

A splice site mutation in the LDL receptor gene has been detected in a homozygous FH patient with the combined use of DGGE and sequence analysis. The mutant alleles inherited by the proband from both parents have an A to G transition in the penultimate 3'-nucleotide of intron 16 of the LDL receptor gene. This transition changed the highly-conserved consensus sequence of the acceptor splice site from AG to GG, thereby abolishing the splicing at the intron 16/exon 17 junction [23]. This is the first report on a naturally-occurring mutation at a 3'-acceptor splice site of the LDL receptor gene. A 5'-donor splice site mutation has been recently characterized by our group [24].

Several 3'-acceptor splice site mutations have been found in other genes [25-29], leading to aberrant splicing and to the activation of cryptic splice sites and/or inclusion of the unspliced intron and, ultimately, resulting in a protein reading frame-shift. cDNA analysis of our mutation showed that a cryptic splice site activated in intron 16, upstream from the original splice site, caused the inclusion of 62 nucleotides, corresponding to the 3'-end of intron 16. The new protein reading frame, showing a TAA stop-codon 204 nucleotides downstream of the normal TGA stop-codon in exon 18, enables us to predict an elongation of about 90 amino acids of the LDL receptor protein, which is consistent with the higher apparent molecular weight of the mutated LDL receptor protein (131 kD), revealed by SDS gel electrophoresis (Fig. 5).

The mutation affects two of the functional domains of the LDL receptor protein: the membrane-spanning domain, encoded by exon 16 and the 5'-end of exon 18 [3]. The immunoprecipitation analysis, which revealed that the monoclonal antibody IgG-C7 recognized the mutant protein, suggested that the mutation did not affect the ligand-binding domain. Binding studies showed that some residual LDL receptor activity is still expressed on the cell surface, whereas internalization and degradation did not occur (Fig. 6). Pulse-chase studies suggested that most of the mutant protein, once synthesized, is not further post-translationally modified, although we cannot completely rule out the possibility that part of it is processed. The main events during intracellular LDL receptor processing are commonly assumed to be the *N*- and *O*-glycosylation of the precursor protein [30]. The main sites in the polypeptide where this post-translational modification takes place are unaltered in the mutant protein. However, secondary structure analysis of the mutant LDL receptor protein, performed according to Chou and Fasman [31], enables to predict that, due to the frame-shift downstream of exon 16 and the consequent elongation, the folding of the mutant protein is highly disrupted. A change of conformation might explain why the mutant protein is not glycosylated. In fact, a correct native protein conformation is a requirement for normal transport of newly synthesized proteins from the endoplasmic reticulum to the Golgi complex [32]. Despite they are incorrectly processed, the mutant receptors appear to be transported to the plasma membrane. Two lines of evidence sustain this observation: firstly, there is residual lipoprotein-binding activity expressed on the cell surface; secondly, the newly

synthesized receptors do not undergo rapid intracellular degradation, as shown by the pulse-chase experiments (Fig. 5).

A change of conformation might also provide an explanation for the reduced binding activity on the cell surface. As recently suggested by Fourie [33], a mutation in the LDL receptor gene that interferes with the folding or post-translational processing of the protein might result in functionally heterogeneous receptors. A second explanation might be that the mutant receptors are not firmly anchored to the plasma membrane, due to the fact that only 8 out of the 21 amino acids originally forming the transmembrane domain are intact.

A number of mutations affecting the same functional domains of the LDL receptors have been described. Among these are the J.D. mutation, a cys → tyr substitution at amino acid residue 807 [34]; the FH Helsinki, exhibiting a deletion of exon 16, 17 and part of 18 [5]; a duplication of exons 16 and 17 [35]; and a duplication of four bases (AGAA) in the region of amino acid residue 796 with a stop codon appearing 8 codons downstream at amino acid 804 [36]. Although these mutations differ greatly on both DNA and protein level, they all lead to the same functional phenotype of internalization-defective LDL receptors. Our data, showing that in MA's fibroblasts the surface binding is not followed by internalization, allow us to conclude that this mutation belongs to the same class. Apparently, the remaining 8 amino acid residues forming the first part of the transmembrane domain, encoded by exon 16, are sufficient to anchor the mutant receptor to the cell membrane, but the disruption of the entire cytoplasmic domain and the unusually long C-terminal amino acid sequence, which has no resemblance to the normal LDL receptor, abolish the signal for clustering in coated pits, thereby preventing the internalization of the ligand-receptor complex.

Acknowledgements

This study was financially supported by The Netherlands Heart Foundation (PL: project # 89.057) and by NWO/HGO/TNO (M.H. project # 900-539-052).

The authors are indebted to Dr. Brian Knight and Dr. Anne K. Soutar for their support in performing the immunoprecipitation experiments and for helpful discussions. Mrs. Marga van Eck is acknowledged for her excellent technical assistance.

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Chapter 8

mRNA DEFICIENCY OF THE LOW DENSITY LIPOPROTEIN RECEPTOR GENE IN HETEROZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA

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Submitted.

Summary

The prevalence of mRNA deficient alleles of the low density lipoprotein receptor (LDLR) gene was investigated in heterozygous familial hypercholesterolemia (FH) patients using the recently developed PCR-based method to study allele-specific transcripts (AST-PCR; Top et al., Biochem. Biophys. Res. Commun. 1991; 178: 1319). This method is based on the assessment of loss of heterozygosity of exon-specific restriction fragment length polymorphisms (RFLPs) at the mRNA level as compared to genomic DNA. Forty-eight out of 74 patients (65%) were informative, i.e. heterozygous, on genomic DNA for at least one of the 4 exon-specific RFLPs used, and were subsequently analyzed at the level of mRNA. In 12 of these 48 samples (25%) allele-specific mRNA deficiency was observed. Haplotype analysis suggests that a limited number of haplotypes may be associated with this mRNA deficiency. Co-segregation between a mRNA deficient LDLR allele and FH was demonstrated in one family. In one patient we analyzed the entire coding sequence of the LDLR gene for the corresponding mutation by a combination of denaturing gradient gel electrophoresis (DGGE) and direct sequencing. Using this approach we could find an insertion of a C at codon 528 in exon 11. This mutation introduces a shift in the reading frame and leads to a stop codon at codon 537. Due to the relatively high frequency of mRNA deficient alleles detectable in the FH patient group analyzed, this assay may provide a useful approach to further the insight in the pathophysiology and thus the diagnosis of FH based on LDLR gene defects.

Introduction

In Western populations familial hypercholesterolemia (FH) affects one in 500 individuals (1). This common autosomal dominant genetic disorder is caused by a variety of mutations in the low density lipoprotein receptor (LDLR) gene (2,3). These LDLR gene mutations can be divided in five classes with respect to their phenotypic effects on the LDL receptor protein caused by defects in: (*i*) LDL receptor synthesis, (*ii*) intracellular transport of LDL receptor, (*iii*) binding of LDL particles, (*iv*) internalization of bound LDL particles and (*v*) dissociation of bound LDL particles in the lysosomal compartment and subsequent receptor recycling (2-4).

Mutations that are impaired in the synthesis of LDL receptor protein, so-called "null" or cross-reacting material negative (crm^-) alleles, are common (5). Null alleles can be further subdivided into: (*i*) those that produce detectable LDLR mRNA and (*ii*) those that fail to produce mRNA ($mRNA^-$). In a collection of 157 fibroblast strains of homozygous FH patients (the so-called "Dallas collection") the frequency of null alleles appeared to be 17%. It was however recognized that this frequency is an underestimate of the actual frequency

because of the fact that more than half of the fibroblast cell lines in this series are in fact derived from compound heterozygotes (3). Cells from compound heterozygotes carry two different LDLR mutations and in fibroblasts of these individuals which produce LDLR protein, it has been difficult to assess whether both alleles produce defective receptors or whether one of the alleles is null (3). Quantitative mRNA measurement by, for instance, Norther blot assays in order to discriminate between normal mRNA expression and heterozygosity for mRNA deficiency is not the method of choice because cultivation of cells from different subjects and the stimulation of LDLR mRNA expression is almost impossible to standardise.

The fact that LDLR mRNA deficient (null) alleles are reported to be relatively common in the FH population (3,5), tempted us to investigate whether detection of these mRNA deficient alleles might be of value for research into underlying molecular defects causing FH and thus for the diagnosis of FH heterozygotes.

To assess allele-specific transcripts we recently developed a PCR-based strategy (AST-PCR) which allows comparison of the relative mRNA levels expressed by the two alleles (6). This method makes use of exon-specific RFLPs to discriminate between mRNA expressed by the two alleles. Based on the assumptions that mRNA expression of the two alleles occurs at an equal rate in the normal situation and that in FH heterozygotes the normal allele fails to compensate for the mutated allele (7), loss of heterozygosity of an exon-specific RFLP at the mRNA level, compared to genomic DNA, is an indication of allele-specific mRNA deficiency.

In the present study AST-PCR was applied to determine the prevalence of allele-specific mRNA deficiency of the LDLR gene in a group of heterozygous FH patients. These experiments reveal that about 25% of the FH heterozygotes carry a mRNA deficient LDLR allele.

Materials and Methods

Subjects

Seventy-four unrelated heterozygous FH patients were included in this study. Patients were attending the Lipid Clinic of the Academic Hospital of the Leiden University or the Lipid Clinic of the Academic Medical Center in Amsterdam. The diagnosis of heterozygous FH was based on the following: (*i*) total plasma cholesterol level > 8 mmol/l, (*ii*) triglycerides < 2 mmol/l, (*iii*) tendon xanthomas and (*iv*) first degree relatives with hypercholesterolemia. Control individuals in this study were normolipidemic volunteers.

DNA and RNA isolations

High molecular weight genomic DNA was isolated from peripheral blood cells as described by Miller (8).

RNA was isolated from growth stimulated, cultured lymphocytes. One ml of plasma depleted blood was added to 10 ml of medium 199 (Gibco) supplemented with 5% fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin, 0.1 mg/ml streptomycin and one vial/200 ml phytohaemagglutinin (Wellcome). Cells were incubated at 37°C for 72 hours and RNA was subsequently isolated using a guanidinium isothiocyanate protocol essentially as described by Chomczynski (9). RNA was dissolved in sterile, diethylpyrocarbonate (DEPC) treated water and stored at -20°C until use. Usually 10-20 μg RNA was obtained.

cDNA synthesis

First strand cDNA was synthesized by incubating 2 μl (approximately 1-2 μg) of RNA with 7.5 units of AMV reverse transcriptase (Pharmacia) in a buffer containing 50 mM Tris.HCl (pH 7.3), 8 mM $MgCl_2$, 30 mM KCl, 2 mM of each nucleotide (dATP, dTTP, dGTP, dCTP) and 10 mM dithiothreitol and 37.5 units RNAguard™ (Pharmacia) in a final volume of 20 μl, at 42°C for one hour. cDNA synthesis was primed with 1 pmol of an exon 18 specific primer; designated as 1834 (Fig. 1).

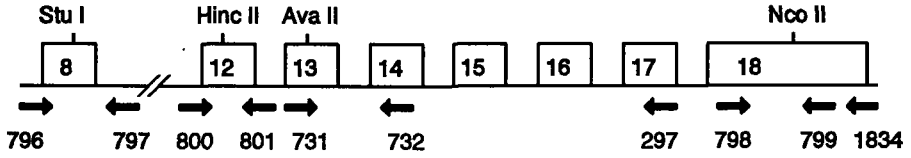

Figure 1. Schematic representation of the localization of the primers used in the amplification of genomic DNA and cDNA fragments of the LDLR gene. Numbered boxes represent the corresponding LDLR exons in which the polymorphic restriction sites are indicated. Specific genomic DNA LDLR gene fragments were amplified for each RFLP. The following primer combinations were used: *StuI* 796/797, *Hinc*II 800/801, *Ava*II 731/732 and *Nco*I 798/799. First strand cDNA synthesis was performed with primer 1834 which is located near the 3'-end of exon 18. Amplification of cDNA fragments is performed with the following primer combinations: 796/801 amplifies a 782 bp fragment to determine both the *StuI* RFLP (S-782 bp, S+ 105/677 bp) and *Hinc*II RFLP (H- 110 bp, H+ 40/70 bp and a constant band of 672 bp); 731/297 amplifies a 555 bp fragment containing the *Ava*II RFLP (A- 120 bp, A+ 50/70 bp and a constant band of 435 bp); 798/799 amplifies a 914 bp fragment containing the *Nco*I RFLP (N- 914 bp, N+ 429/485 bp). Exons and introns are not drawn to scale.

Polymerase chain reaction (PCR)

Amplification of genomic DNA and cDNA fragments was carried out in a buffer containing 10 mM Tris.HCl pH 8.4, 50 mM KCl, 200 μM of each dNTP, 2.5 mM $MgCl_2$, 50 pmol of each primer (see Fig. 1) and 1 unit Taq-polymerase (Amplitaq, Perkin Elmer) in a final volume of 50 μl. Primers (Table 1) were synthesized on a Biosearch Cyclone™ DNA synthesizer. The PCR was performed on 500 ng genomic DNA or 10% of the first strand cDNA synthesis mixture at 1 minute 94°C (denaturation), and 6 minutes 65°C (annealing and elongation) for 32 cycles and 45 cycles, respectively, using an automated thermal cycler

Table 1. Primers for PCR and reverse transcription.

number	location	sequence (5'-3')
297	exon 17	CCGCCAGTTCTTCCATAGAA
731	exon 13	AGTGCCAACCGCCTCACAGG
732	exon 14	CCTCTCACACCAGTTCACTC
796	exon 8	ATCGATGAGTGTCAGGATCC
797	intron 8	CCCGCCGCCTTCCCGTGCTGA
800	exon 12	ATCTCCTCAGTGGCCGCCTC
801	exon 12	CTCAAAGACGGCCAAGGAGA
798	exon 18	CACCTAGTGCTTCCACTTCTA
799	exon 18	TCCCGTCAAACGATCCAGACT
1834	exon 18	CACTGAACAAATACAGCAAC

(Biomed 60). Primer combinations used for the amplification of cDNA and genomic DNA fragments are shown in Fig. 1. To prevent amplification of traces of contaminating genomic DNA in the RNA preparation, primers to specifically amplify LDLR cDNA fragments were chosen in such a way that the fragments span multiple exons which are interrupted by introns in the corresponding genomic DNA (Fig. 1). However, the *Nco*I polymorphism is located at the 3'-end of exon 18 which is 2.5 kb in size and therefore identical primers were used to amplify genomic and cDNA. In order to monitor contaminating genomic DNA, 2-4 μg of total RNA, which is ten times more than the amount used in a typical reaction, was amplified for 45 cycles with primers 798/799. No fragments were visible on an agarose gel indicating that the RNA was not detectably contaminated with genomic DNA (data not shown).

RFLP analysis of amplified DNA fragments

The specificity of the PCR products of genomic DNA and cDNA was examined by electrophoresis of 5 μl aliquot on 1.5% agarose gel (10). For RFLP analysis 5 μl of amplified DNA fragments were digested with 3 units of the appropriate restriction endonuclease under conditions recommended by the manufacturer (Pharmacia), size fractionated on either 2% agarose gel (*Ava*II, *Stu*I, and *Nco*I), or 10% non-denaturing polyacrylamide gels (*Hinc*II) and visualized by ethidium bromide staining. For the amplified cDNA fragments *Nco*I and *Stu*I RFLPs were determined on a 2% agarose gel. *Ava*II and *Hinc*II fragments were subsequently size separated on 10% non-denaturing polyacrylamide gels, transferred to Hybond N^+ membranes (Amersham) by electroblotting, hybridized with an exon 10 to 14 containing LDLR cDNA probe (11) and visualized by autoradiography on Kodak XAR-5 films using an intensifying screen (Konica).

RFLP detection by Southern blot hybridization

For the detection of the RFLPs recognized by the restriction endonucleases *Bsm*I, *Pvu*II and

*Sph*I, 8 μg of genomic DNA was digested with the respective enzymes in buffers as recommended by the manufacturer (Pharmacia). After electrophoresis of the digested DNA fragments through a 0.7% agarose gel the DNA fragments were transferred by alkaline blotting to Hybond N^+ (Amersham). Hybridisation with radiolabelled probes was carried out under conditions as described previously (11). The *Bsm*I RFLP was detected with probe mLDLRp1 (12) containing a 552 base pair fragment of the LDLR promoter region and exon 1 while the *Pvu*II RFLP was detected with probe pr34, containing exon 10 to 14 of the LDLR gene (11).

Denaturing gradient gel electrophoresis (DGGE) and direct sequencing

For mutational analysis of the entire coding sequence of the LDLR gene, we applied denaturing gradient gel electrophoresis of GC-clamped PCR fragments of each individual LDLR exon. The strategy and the experimental details of this gene scanning strategy will be reported elsewhere (P. Lombardi, manuscript in preparation). Briefly, each LDLR exon, including the exon/intron junctions, was amplified by PCR with intron-specific primers. A 50 base pair CG-clamp, functioning as the highest melting domain, was attached to the fragments at either the 5'- or 3'-side by a second round of PCR as described previously (13). Perpendicular gel electrophoresis and travel schedule experiments were performed to determine the optimal denaturing gradient and electrophoresis conditions for each fragment.

Exon 11 was amplified with the following intron specific primers; 2713 (5'-*CGCCGCCGCCGCCCG*CAGCTATTCTCTGTC-3'; GC-stretch in italics) and 2714 (5'-TGGCTGGGACGGCTGTCCT-3'). The PCR conditions were the same as described above. 1 μl of the PCR product was subjected to a second round of PCR to elongate the GC-clamp using primer 2714 and a standard 50 base pair GC-rich primer with 3'-homology to 2713 (13). The resulting GC-clamped PCR fragment was analyzed on a 9% polyacrylamide gel containing a 35-65% linearly increasing denaturing gradient. DGGE was performed overnight at 60°C at 6 Volt.cm^{-1}. After electrophoresis gels were stained with ethidium bromide and examined under UV illumination.

Direct sequencing of exon 11 of FH-874 was performed on biotinylated PCR products generated by primers 2713 and 2714-bio. The PCR product was purified from the reaction mixture with streptavidin coated paramagnetic beads as recommended by the manufacturer (Dynal). One-tenth of the purified fragment was subsequently used for the sequencing reaction using the *AmpliTaq* cycle sequencing kit (Perkin Elmer Cetus) using primer 2713-end labelled with [^{32}P]-dCTP (10 mCi/ml, Amersham). The cycle sequencing protocol included 20 cycles for 1.5 minutes at 95°C and for 2 minutes at 65°C in a volume of 8 μl. Sequencing gels were 6% polyacrylamide containing 8 M urea and electrophoresis was carried out at 60 Watt for 2 hours (about 3500 Volt.hours). Gels were exposed to Kodak X-Omat films for 16 hours.

Results

The detection of allele-specific mRNA deficiency by means of AST-PCR is based on the detection of loss of heterozygosity of exon-specific RFLPs in amplified cDNA fragments as compared to genomic DNA (6). Reliable detection of loss of heterozygosity at the cDNA level infers that no preferential PCR amplification of cDNA fragments of one of the two alleles occurs. Figure 2 shows a *Hinc*II RFLP analysis on amplified cDNA of five control individuals which were heterozygous at the DNA level. The relative intensities of the hybridisation signals of the two allelic fragments (H- and H+) are identical in all samples. The absence of variability in the relative intensities of the allele-specific fragments in control samples supports the conclusion that aberrant relative intensities of allelic fragments in patient samples will not be due to PCR artefacts but rather to different starting amounts of the allele-specific mRNA. Therefore, allele-specific mRNA deficiency may be scored not only if one of the alleles is completely absent (complete loss of heterozygosity) but also if the ratio of the intensities of the alleles differs from that in control samples (partial loss of heterozygosity) (6).

Currently, more than 20 polymorphic restriction sites are known in the LDLR gene (14). However, most of these are located in the non-coding regions of the gene and can therefore not be used in the AST-PCR procedure (6). In this study the four exon-specific polymorphisms *Stu*I (exon 8), *Hinc*II (exon 12), *Ava*II (exon 13) and *Nco*I (exon 18) were used.

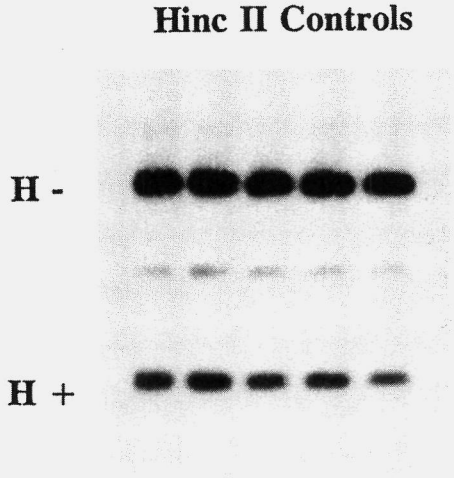

Figure 2. *Hinc*II RFLP analysis on amplified cDNA fragments of five control individuals, heterozygous for *Hinc*II on genomic DNA, demonstrating identical relative intensities of the allelic fragments. H- and H+ denote allelic fragments corresponding to absence or presence of the *Hinc*II restriction site. The shortest H+ fragment has run out of the gel.

Table 2. Genotyping data and cumulative heterozygosity of LDLR gene RFLPs.

	genotype (n = 74)			cumulative heterozygosity	
RFLP	+-	--	++	n	%
Ava II	40	19	15	40	54
Hinc II	40	15	19	40	54
Stu I	6	1	67	41	55
Nco I	38	5	31	48	65

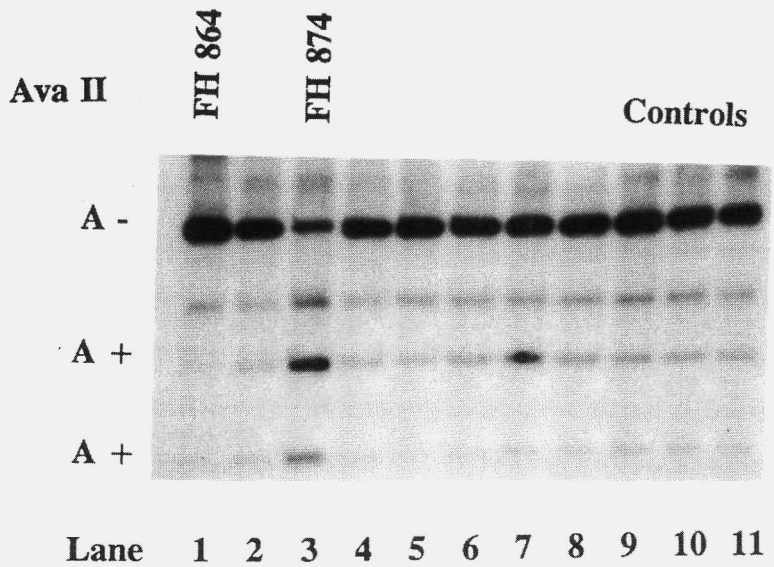

Figure 3. *Ava*II RFLP analysis on amplified cDNA fragments of nine FH patients (lanes 1-9) and two control samples (lanes 10 and 11) showing abnormal relative intensities of the allelic fragments in two patient samples (FH 864 and 874). A- and A+ denote allelic fragments corresponding with absence or presence of the *Ava*II restriction site.

Because heterozygosity for these RFLPs is essential for this method, we started to determine the actual genotypes of the 74 FH patients. For each RFLP a specific genomic DNA fragment was enzymatically amplified by PCR, using primers flanking the polymorphic restriction site (Fig. 1), digested with the appropriate restriction endonuclease and subsequently analyzed by gel electrophoresis. The observed genotypes and the cumulative heterozygosity of the four RFLPs tested are summarized in Table 2. The allele frequencies of each RFLP in our patient population are comparable to those previously reported (14).

Forty-eight out of seventy-four individuals (65%) were heterozygous for at least one RFLP. The remaining 35% of the patients were not informative for any RFLP and therefore not analyzed further. It should be noted that the *Ava*II and *Hinc*II RFLPs are in complete linkage disequilibrium with each other; all samples homozygous for the presence of the *Ava*II restriction site (++) were homozygous for the absence of the *Hinc*II restriction site (--).

Following DNA genotyping, each of the informative markers was then analyzed at the mRNA level. RNA was isolated from growth stimulated cultured lymphocytes and prior to PCR, converted to cDNA by reverse transcription using an exon 18 specific primer.

An example of a typical *Ava*II RFLP analysis is shown in Fig. 3. cDNA of nine FH heterozygotes and two controls was amplified with primers 731/297 and digested with *Ava*II. The polymorphic fragments (A- and A+) were visualized by hybridisation with a LDLR cDNA probe. Seven out of these nine patient samples (lanes 2, 4-9) show an identical relative intensity of the bands and are comparable with the control samples (lanes 10 and 11). Two samples show an aberrant relative intensity of the allelic fragments. FH-864 shows a less relative intense A+ allele (lane 1), whereas FH-874 shows a less relative intense A- allele (lane 3) indicating that FH-864 has a mRNA deficiency corresponding with the A+ allele and FH-874 with the A- allele.

Upon screening all 48 informative individuals, partial loss of heterozygosity for at least one RFLP was observed in 12 individuals. Figure 4 gives an overview of the hybridisation pattern (*Ava*II and *Hinc*II) and ethidium bromide staining patterns (*Stu*I and *Nco*I) of those samples showing reduction of one of the allelic fragments as compared to normal controls. Ten out of twelve samples showed loss of heterozygosity with *Ava*II (panel A). Four of these showed loss of the A+ allele (lanes 4, 5, 9 and 10) whereas six showed loss of the A- allele (lanes 1, 2, 3, 6, 7 and 8). Because of the linkage disequilibrium between *Ava*II and *Hinc*II, these samples also showed loss of the respective H- allele (4 out of 10) or H+ allele (6 out of 10) (panel B). Five of these ten samples were also informative with *Nco*I and, indeed, showed loss of heterozygosity using this enzyme (panel C; lanes 2, 3, 5, 6 and 8). Two additional samples that were not informative for *Ava*II and *Hinc*II, showed loss of heterozygosity with *Nco*I (panel C; FH-808 and FH-882, lanes 1 and 7, respectively). No loss of heterozygosity with *Stu*I was observed in this group. Combining the results of the RFLP analysis of these four markers, we constructed haplotypes of the mRNA deficient LDLR allele in 12 samples that showed loss of heterozygosity (Table 3). Three different haplotypes, designated A, B and C, could be distinguished and each of these haplotypes was found in 4 out of 12 samples.

To demonstrate a direct relationship between the observed mRNA deficiency and the clinical diagnosis of FH, the cosegregation of the mRNA deficient allele (haplotype B) and the FH phenotype was analyzed in one family (Fig. 5). In this family mRNA deficiency was initially observed in individual II-4 (FH-906 in Fig. 4). Five other family members who were diagnosed as FH based on plasma cholesterol levels (I-2, II-2, II-6, III-2 and III-3) inherited the haplotype which is associated with the mRNA deficiency. Three additional intron-specific

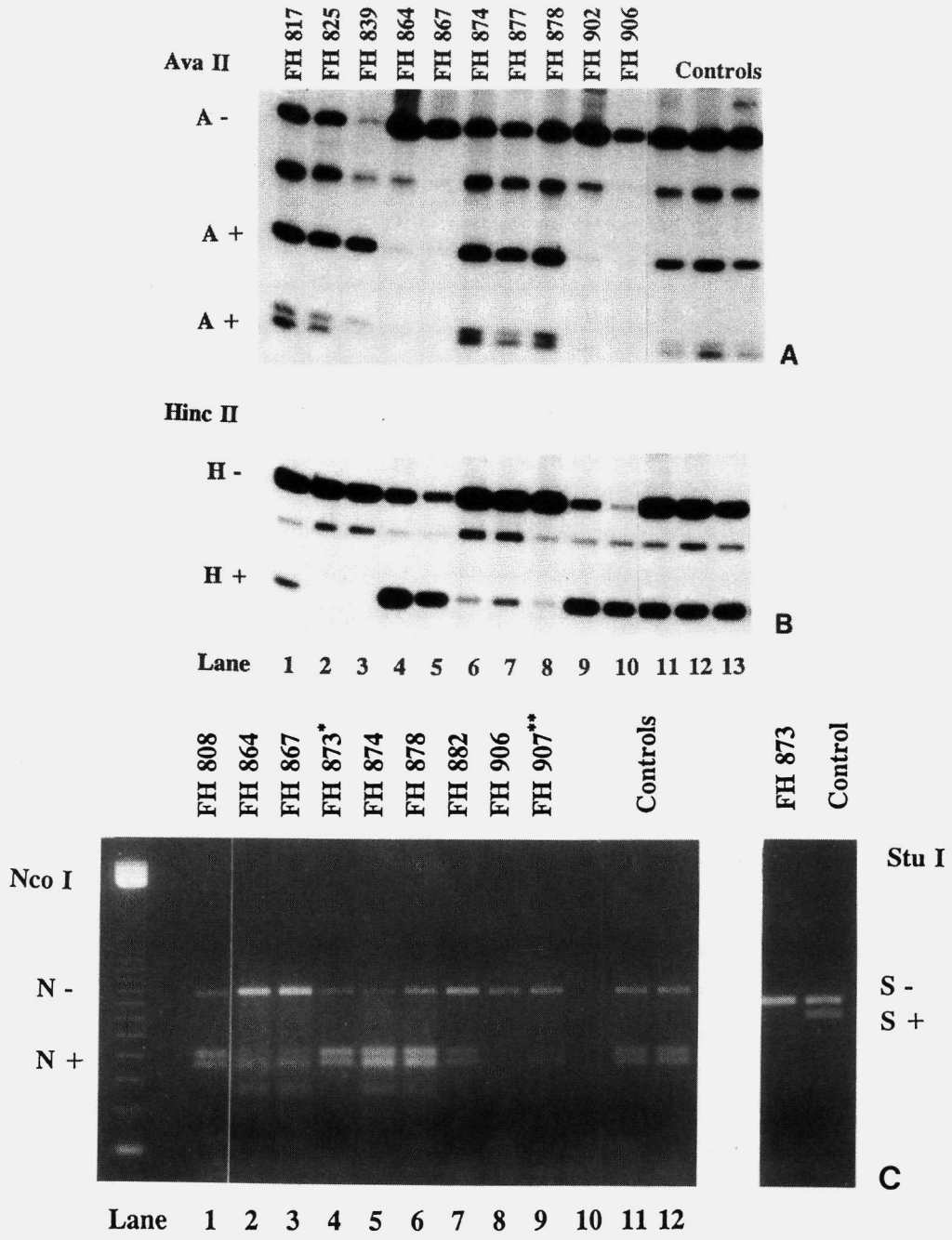

Figure 4. Hybridisation pattern (*Ava*II and *Hinc*II; panel A and B) and ethidium bromide staining pattern (*Stu*I and *Nco*I; panel C) of those samples showing loss of one of the allelic fragments on amplified cDNA fragments as compared to heterozygous normal control samples. * and ** FH-873 and FH-907 represent patients which are not included in the original patient group (n = 74) but are relatives of FH-874 and FH-906, respectively. The most left lane in panel C is a 123 base pair DNA ledder (Gibco, BRL).

Table 3.

	DNA genotype				mRNA deficient allele				Haplotype
patient	*Ava* II	*Hinc* II	*Stu* I	*Nco* I	*Ava* II	*Hinc* II	*Stu* I	*Nco* I	
FH 808	--	++	++	-+	-	+	+	-	A
FH 817	-+	-+	++	++	-	+	+	+	C
FH 825	-+	-+	++	++	-	+	+	+	C
FH 839	-+	-+	++	++	-	+	+	+	C
FH 864	-+	-+	++	-+	+	-	+	+	B
FH 867	-+	-+	++	-+	+	-	+	+	B
FH 874	-+	-+	++	-+	-	+	+	-	A
FH 877	-+	-+	++	--	-	+	+	-	A
FH 878	-+	-+	++	-+	-	+	+	-	A
FH 882	--	++	++	-+	-	+	+	+	C
FH 902	-+	-+	++	++	+	-	+	+	B
FH 906	-+	-+	++	-+	+	-	+	+	B

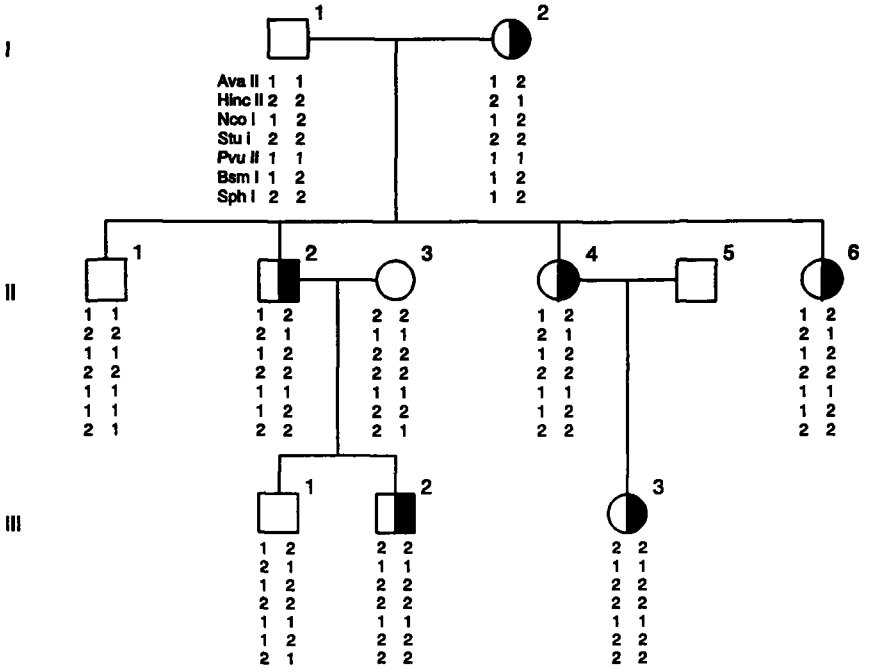

Figure 5. Pedigree of the family of FH-906 (II-4) demonstrating cosegregation between the mRNA deficient haplotype (2122122) and the FH phenotype. Half filled symbols represent heterozygous FH patients as based on plasma cholesterol levels. 1 and 2 correspond to the absence (-) or presence (+) of the respective restriction site.

RFLPs (*Bsm*I, *Pvu*II and *Sph*I) were determined by Southern blot analysis to extend this haplotype. mRNA deficiency was confirmed in individual II-6 (also designated as FH-907) with *Ava*II, *Hinc*II (not shown) and *Nco*I (Fig. 3, lane 9).

In order to find the underlying mutations causing the allele-specific mRNA deficiency, we applied denaturing gradient gel electrophoresis (DGGE) of each exon of the LDLR gene. Each of the LDLR exons including the exon/intron boundaries were amplified using intron-specific primers. To ensure that each fragment is suitable for DGGE a 50 base pair universal GC-clamp was attached to the fragments using a two-step PCR protocol (13). Using this approach, we found that the mutation in FH-874 was located in exon 11. Sequence analysis of this exon revealed an insertion of a C at codon 528 resulting in a shift of the reading frame and the introduction of a premature stop codon at codon 537 (Fig. 6). The insertion of the C at this position also creates a new *Hinf*I restriction site. Restriction analysis of the PCR product of exon 11 not only confirmed the presence of this mutation but also that this mutation was present in a relative of FH-874 (FH-873, not shown) having an identical mRNA deficient haplotype (Fig. 4). No other mutations were found in any of the other exons in this patient.

Discussion

Analysis of fibroblasts obtained from homozygous FH patients has revealed that a considerable fraction of the mutations of the LDLR gene results in a reduction of steady-state LDLR mRNA levels (5). Here, we have determined the frequency of mRNA deficient alleles using a PCR-based method (6) in a group of 74 heterozygous FH patients. To study mRNA deficiency four exon-specific LDLR gene RFLPs (*Stu*I, *Hinc*II, *Ava*II and *Nco*I) were used to monitor loss of heterozygosity at the mRNA level as compared to genomic DNA. Among 74 patients, 48 had an informative genotype for at least one RFLP and were subsequently analyzed on amplified cDNA fragments. In 12 out of these 48 samples (25%), loss of heterozygosity was detected. Taking into account the non-informative cases (35% of the patients in our group were homozygous for all four RFLPs), eventually mRNA deficiency could be scored in 12 out of 74 cases (16%). To improve this frequency, additional RFLPs, like *Msp*I in exon 18 (14) and the dinucleotide TA(n) repeat in exon 18 (15), may be included as well. In all these 12 cases residual amounts of mRNA of the deficient allele was present, indicating that the mRNA expression of the mutated allele was not completely abolished.

Several lines of evidence supports the hypothesis that the observed allelic loss of mRNA is due to different relative amounts of steady-state mRNA levels of the two alleles rather than to preferential amplification of cDNA of one of the two alleles or other PCR artefacts: (*i*) differences in relative intensities of the two allelic fragments were never observed in control samples; (*ii*) in most samples allele-specific mRNA deficiency was detected with at least two

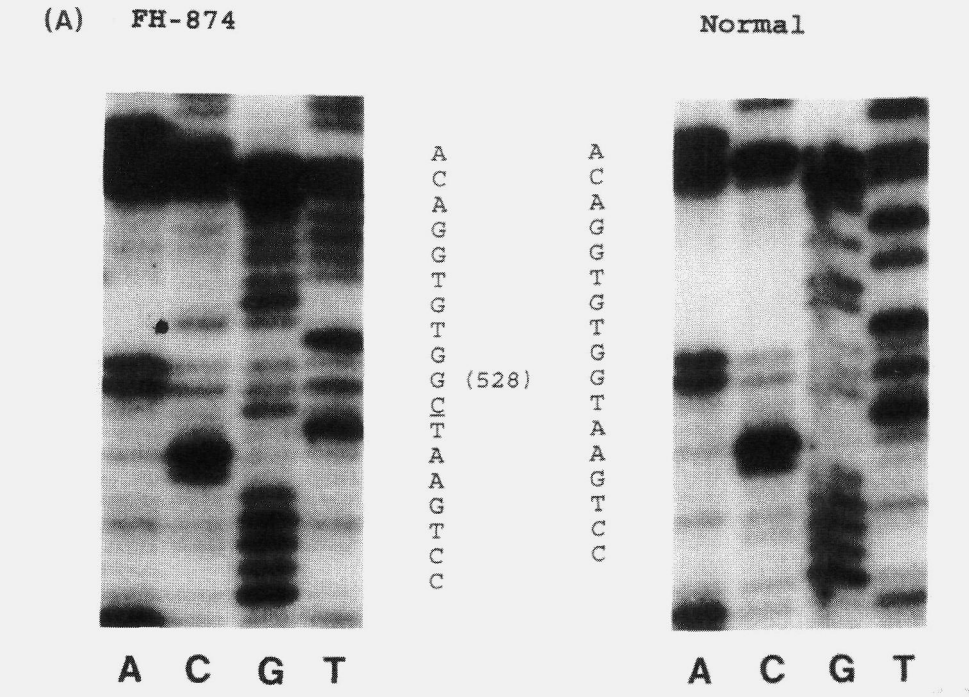

Figure 6. Partial sequence of the sense strand of exon 11 of FH-874 showing the C-insertion at codon 528 (marked with a dot; panel A). The mutation causes a shift in the open reading frame and, ultimately, leads to the introduction of a new stop codon at amino acid 537 (panel B).

different combinations of RFLP and amplified fragment; (*iii*) a haplotype associated with allelic loss of mRNA cosegregates with FH in a family; (*iv*) similar results were obtained when the reverse transcription was carried out with other exon 18 specific primers excluding the possibility of allele-specific mispriming (not shown).

An interesting question that follows concerns the molecular defect that is responsible for the observed allelic loss of LDLR mRNA in the patients described here. Previous reports have shown that large deletions that comprise the promoter region and the 5'-part of the LDLR gene in FH patients cause complete absence of gene transcription (5,16,17). We have performed Southern blot analysis of the FH patients included in this study using cDNA probes covering the entire LDLR gene and the promoter region (now shown), but no large gene rearrangements were present.

From this result we may conclude that the reduced levels of mRNA of the LDLR gene could be the result of a small (point) mutation that either reduces transcriptional activity or affects stability of mRNA. With respect to the transcriptional activity, we have previously reported a screening for point mutations in the regulatory elements in the promoter region of the LDLR gene in a population of 350 FH patients using denaturing gradient gel electrophoresis. We concluded that mutations were absent in this part of the gene and consequently do not play a role in the etiology of FH (12). Thus, combining the data from the Southern and DGGE analysis, it is reasonable to speculate that mutations affecting mRNA abundance in the FH patients described in this report are most likely located either outside presently known promoter sequences or located within the coding sequence of the gene and affect mRNA stability. To scan the entire coding sequence of the LDLR gene for these kind of mutations, we have set up a strategy which involves GC-clamped PCR, denaturing gradient gel electrophoresis and solid phase cycle sequencing. Using this approach we were able to characterize the mutation in one of the FH patients (FH-874) as an insertion of a C at codon 528 in exon 11. This particular mutation induces a shift in the reading frame and results in a premature stop codon at position 537. For this patient no other mutations were found in the other LDLR exons. Translation of this mRNA will lead to a non-functional LDL receptor protein since it lacks almost 330 C-terminal amino acids which in the normal receptor form several critical functional domains such as the transmembrane and the transmembrane and cytoplasmic domains.

Apart from the functional consequences on the LDLR protein of this mutation it is interesting to know whether this mutation is the cause of the mRNA reduction. In favor of this role are several reports that have demonstrated that mutations that introduce a premature termination of translation are associated with decreased intracellular levels of the mutant mRNA (18-22). The exact mechanism by which mRNA abundance is affected by premature termination codons is not known. It has been suggested that these codons may reduce the steady-state level of mRNA by inhibiting the processing of downstream exons (23), leading to an abnormal polyribosome content, leaving an unprotected mRNA segment which is amenable to degradation (24). In the human LDLR gene two mutations have been described that are associated with low mRNA levels (2,3). For example, the mutations designated *FH-Turkey* and *FH-Nashville* generate nonsense mutations in exon 2 and 8, respectively. In addition, Hummel et al. (25) demonstrated that in hypercholesterolemic rhesus monkeys a nonsense mutation in exon 6 of the LDLR gene severely reduces the mRNA level.

Using ten RFLPs distributed over the entire LDLR gene, Leitersdorf and coworkers found that in a normal population 31 haplotypes of the LDLR gene exist

(26). Three of these haplotypes (designated 1, 2 and 3) occur with a combined frequency of 60%. The remaining haplotypes are less frequent and have a population frequency between 0.8% and 4.9%. It would be of interest to determine if haplotypes correlated with mRNA deficiency occur more frequently in a FH population than in a normal population. In the mRNA deficient FH patient group, three different haplotypes (A, B and C) could be deduced with the four RFLP markers tested. Comparison of these haplotypes with those reported by Leitersdorf et al. (27) showed that each of these three haplotypes can be further subdivided in at least six haplotypes by using additional RFLPs. Extended haplotyping of FH families with mRNA deficient alleles will eventually elucidate the number and frequency of different haplotypes that are associated with mRNA deficiency.

Despite the fact that Southern analysis has revealed a large number of different gene rearrangements in the LDLR gene in various populations, the relatively low frequency and heterogeneity of these kind of mutations has hampered the use of this technique for the identification of individual FH patients. Due to the relatively high frequency of mRNA deficient alleles detectable in the FH patient group analyzed, this assay may provide a useful approach to further the insight in the pathophysiology and thus the diagnosis of FH based on LDLR gene defects.

Acknowledgements

We wish to thank Drs. P.J. Lansberg and B. Groenemeier for their help in obtaining patient material; L. van Mourik for performing the pedigree analysis and Drs. M.H. Hofker and A.M.J.M. van den Maagdenberg for their helpful discussions. This work was supported by the Health Research Division TNO.

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Chapter 9

THE PRESENCE OF SEQUENCE VARIATIONS IN THE LOW DENSITY LIPOPROTEIN RECEPTOR GENE OF FAMILIAL HYPERCHOLESTEROLEMIC PATIENTS DETECTED BY DENATURING GRADIENT GEL ELECTROPHORESIS

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Submitted.

Summary

Classical Familial Hypercholesterolemia (FH) results from mutations in the Low Density Lipoprotein Receptor (LDLR) gene. We applied Denaturing Gradient Gel Electrophoresis (DGGE) to screen for sequence variations in the coding and splice site consensus sequences of the LDLR gene in genomic DNA. For amplification of each exon by the polymerase chain reaction (PCR) optimal pairs of primers were designed by the MELT 87 computer algorithm. To optimize the ability to detect mutations one primer of each set was extended with a GC-clamp. DGGE screening of amplified DNA from 32 apparently unrelated heterozygous FH patients revealed unique sequence variations in 27 patients. Among these, six were located in exon 4, five in exon 3, four in exon 6, three in exons 7, 10 and 12 respectively, and one in exons 2, 9, 11 and 15, implying a high score in the binding region (16 out of 27). In addition to these unique mutations, all seven known exon-bound polymorphisms were detected. Regardless these neutral polymorphisms, 26 subjects showed a sequence variation only in one LDL receptor allele; one patient had sequence variations in both alleles. In a group of 32 normal subjects, all the common polymorphisms could be unequivocally detected by DGGE, while none of the sequence variations identified in the FH patients was present, suggesting that these are likely to be putative candidates for causing the disease. These results indicate that DGGE provides an excellent strategy for detection of the presence of disease-causing mutations and common polymorphisms in the LDL receptor gene.

Introduction

Classical familial hypercholesterolemia (FH) is an autosomal dominantly inherited disease caused by mutations in the gene encoding for the Low Density Lipoprotein Receptor (LDLR), which mediates the specific uptake of plasma LDL. In most populations, the heterozygous form of FH is common, affecting approximately one person in every five hundred. Heterozygous FH individuals have only half the normal number of functional LDL receptors and, as a consequence, plasma LDL cholesterol levels of about twice the normal level, leading to an increased risk of premature atherosclerosis. Homozygous FH patients are more severely affected and rarely reach the age of maturity (1). Homozygous FH is rare and clinically unmistakable, while it may not be possible to make an unequivocal diagnosis of heterozygous FH, unless the presence of tendon xanthomas or a well-defined family history of hypercholesterolemia has been recorded.

After the LDL receptor gene was cloned (2), a vast array of mutations including insertions, deletions, nonsense and missense mutations have been described, affecting either the synthesis, post-translational processing, ligand binding activity or internalization of the LDL receptor (3). Most mutations so far characterized appeared to be unique, except for

those cases, in some isolated populations, where a founder effect has resulted in gene enrichment (4-7).

So far, 150 different mutations have been identified in the LDLR gene of FH patients (3). Among these, one-third consists of major structural rearrangements detectable by Southern blotting (3,8-10). The remaining mutations are predominantly point mutations or small insertions or deletions (3). The marked heterogeneity of FH has so far precluded the development of simple screening tests for the identification of mutations in the LDL receptor gene. Such tests may facilitate studies on the genotype-phenotype relationship and the effect of treatment.

In recent years, several reports have shown that Denaturing Gradient Gel Electrophoresis (DGGE) is a powerful method for the near complete detection of the presence of disease-related mutations and polymorphisms (11-13). In the present study, we report the development of a GC-clamped DGGE assay that enables an efficient screening of the coding and splice-site consensus sequences of the LDL receptor gene for the presence of sequence variations. In a group of 32 apparently unrelated heterozygous FH patients, we identified the previously reported common polymorphisms (14-20) and twenty-eight sequence alterations none of which has been found in normal controls, implying that these mutations are most likely to be responsible for FH.

Materials and Methods

Subjects

Thirty-two patients were examined in this study. They were randomly selected from a collection of apparently unrelated patients with a clinical diagnosis of heterozygous FH, attending the Lipid Clinic of the Academic Hospital of Leiden University. The selection criteria applied were: (*i*) plasma cholesterol concentration above 9.5 mmol/l after 9 weeks of diet and without cholesterol-lowering medication, (*ii*) triglycerides < 2 mmol/l, (*iii*) presence of tendon xanthomas or (*iv*) family history of coronary artery disease, (*v*) absence of structural rearrangements in the LDL receptor gene by conventional Southern analysis performed as previously described (10), (*vi*) absence of the B3500 gene, characteristic of familial defective apolipoprotein B (FDB) (21).

Control individuals were healthy normolipidemic volunteers from the RIFOH study (22).

DNA isolation

Genomic DNA was isolated from peripheral leukocytes, according to the method of Miller et al. (23).

Melting map predictions

Melting maps of each genomic (exon) fragment with a GC-clamp attached to either the 5'-

Table 1. Oligonucleotide primers for PCR amplification of individual exons of the human LDL receptor gene.

Exon	5'-primer (5' → 3')	3'-primer (5'→ 3')
1:	B[a] 2772[b] CGCCGCCGCCGCCCGAATGCTGTAAATGACGTGG[c]	A 2773 TTCTGGCGCCTGGAGCAAG
2:	B 3118 GCCGCCGCCGCCCGCCTTTCTCCTTTTCCTCTCTCTC	A 3119 AAAATAAATGCATATCATGCCCA
3:	B 3078 CGCCGCCGCCGCCCGTGACAGTTCAATCCTGTCTCTTC	A 3079 AATAGCAAAGGCAGGGCCACACT
4:	A 2534 TGGTCTCGGCCATCCATCC	B 2535 CGCCGCCGCCGCCCGACGCCCCGCCCCCACCCTG
5:	A 2536 CAACACACTCTGTCCTGTT	B 2537 CGCCGCCGCCGCCCGGGGAAAACCAGATGGCCAGC
6:	B 2668 CGCCGCCGCCGCCCGTCCTTCCTCTCTCTG	A 2669 GCAAGCCGCCTGCACCGAG
7:	B 3120 CGCCGCCGCCGCCCGAGTCTGCATCCCTGGCCCTGCGC	A 3121 AGGGCTCAGTCCACCGGGGAATC
8:	B 2467 CGCCGCCGCCGCCCGCCAAGCCTCTTTCTC	A 2468 CCACCCGCCGCCTTCCCGT
9:	B 1205 CGCCGCCGCCGCCCGGGCTGCAGGCAGGGGCGACG	A 1289 CTGACCTCGCTCCCCGGACC
10:	A 2715 ATGCCCTTCTCTCCTCCTG	B 2716 CGCCGCCGCCGCCCGAGCCCTCAGCGTCGTGGAT
11:	B 2713 CGCCGCCGCCGCCCGCAGCTATTCTCTGTC	A 2714 TGGCTGGGACGGCTGTCCT
12:	B 2469 CGCCGCCGCCGCCCGTCTCCTTATCCACTT	A 2470 TTCGATCTCGTACGTAAG
13:	B 3122 CGCCGCCGCCGCCCGGTCATCTTCCTTGCTGCCTGTTT	A 3123 GTTTCCACAAGGAGGTTTCAAGG
14:	B 2717 CGCCGCCGCCGCCCGCCTGACTCCGCTTCT	A 2718 ACGCAGAAACAAGGCGTGT
15:	A 3124 AGAAGACGTTTATTTATTCTTTC	B 3125 CGCCGCCGCCGCCCGGTGTGGTGGCGGGCCCAGTCTTT
16:	B 2770 CGCCGCCGCCGCCCGCCTCACTCTTGCTTC	A 2771 CGCTGGGGGACCGGCCCGC
17:	B 2768 CGCCGCCGCCGCCCGTGACAGAGCGTGCCTC	A 2769 TGGCTTTCTAGAGAGGGTC
18:	B 2774 CGCCGCCGCCGCCCGTCCGCTGTTTACCATT	A 2775 TCTCAGGAAGGGTTCTGGG
GC clamp:	C 2548 CGCCCGCCGCGCGCCGCGCCCGTCCCGCCGCCGCC-CGCCGCCGCCGCCCG	

[a] designation

[b] code

[c] The underline represents the 15 bp GC-rich sequence that acts as a linker

or the 3'-primer were generated with the MELT87 computer algorithm (24). MELT87 calculates the melting temperature (Tm) of a given DNA sequence as a function of its nucleotide sequence and composition.

Amplification of the exons of the LDL receptor gene by the polymerase chain reaction (PCR)
PCR primers, homologous to the intron sequences flanking the 18 exons of the LDL receptor gene, were designed on the basis of the sequences published by Leitersdorf et al. (6). Primers were synthesized on a Bioresearch Cyclone™ synthesizer. A list of primer sequences is given in Table 1. To optimize the resolution power of DGGE, a two-step PCR protocol was developed to obtain amplification products with an artificial high-melting domain (GC-clamp) (25,26). An outline of the PCR strategy is represented in Fig. 1. One primer of each set (primer B) contains at the 5'-end a GC-sequence of 15 nucleotides that acts as a linker. The primer which did not contain the GC-rich sequence was indicated as A. The 15 bp GC-rich sequence was further elongated to a 50 bp GC-rich sequence in a second PCR run, using primer C, a 50-mer that has, at its 3'-end, a sequence identical to the GC-stretch in primer B. The remaining 35 bp of this primer are random Gs and Cs. In general, PCR was carried out on approximately 500 ng of genomic DNA in a buffer containing 10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM of each deoxy nucleoside 5'-triphosphate (dNTP), 50 pmol of each primer and 1 unit of *Taq* DNA polymerase (Amplitaq, Cetus, Norwalk, Conn.) or 0.2

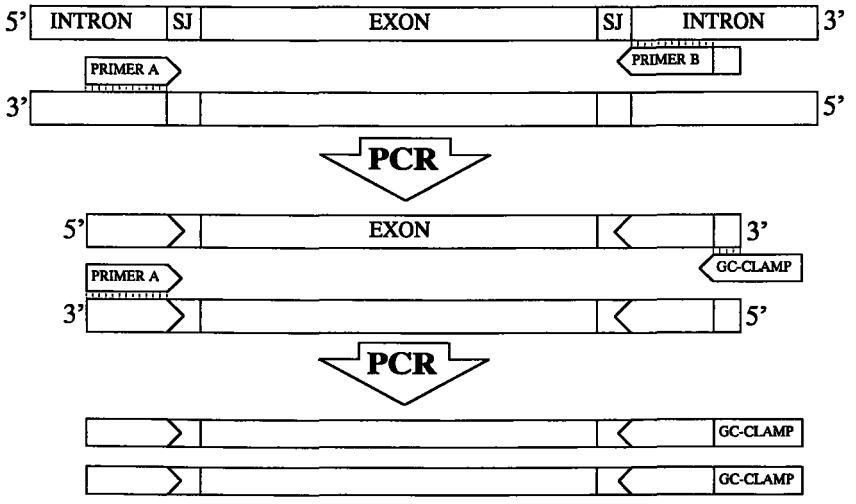

Figure 1. Outline of PCR strategy. In the first PCR step, genomic DNA is amplified with intron-specific primers, denominated A and B. Primer B contains a GC-rich sequence of 15 nucleotides, which acts as a linker for primer C. In the subsequent PCR, the 15 bp GC-rich sequence is further elongated to a 50 bp GC-rich sequence using primer C, a 50-mer primer that contains a 15 bp GC-rich sequence, homologous to the one present in primer B. SJ denotes the splice junction. The figure is not to scale.

Table 2. PCR conditions for amplification of the individual exons of the LDLR gene.

Exon	DMSO[a]	ST[b]/AT[c]	$MgCl_2$ (mM)	Denaturing gradient (%)[d]
1	-	ST	1.5	60-80
2	+	ST	1.5	40-75
3	+	AT	1.5	40-75
4	+	AT	1.0	50-80
5	+	ST	1.5	50-75
6	+	ST	1.5	40-70
7	-	AT	1.0	50-80
8	-	AT	1.0	50-80
9	+	AT	1.0	50-70
10	+	ST	1.5	45-75
11	-	ST	1.5	35-65
12	-	ST	1.5	35-75
13	-	ST	1.5	35-70
14	-	ST	1.5	40-70
15	-	ST	1.5	40-80
16	-	ST	1.5	55-80
17	-	ST	1.5	35-65
18	-	ST	1.5	50-80

[a] + and - indicate, respectively, the absence or presence of DMSO.
[b] ST indicates *SuperTaq* (HT Biotechnology, U.K.).
[c] AT indicates *AmpliTaq* (Cetus, Perkin Elmer).
[d] 100% denaturant is equivalent to 7 M urea and 40% (v/v) deionized formamide.

unit of *Taq* DNA polymerase (SuperTaq, HT Biotechnology, U.K.). Experimental details, including DMSO and $MgCl_2$ concentrations, are given in Table 2. The amplification protocol consisted of 1 minute denaturation at 94°C, 30 seconds annealing at 55°C and 90 seconds extension at 72°C for 32 cycles. Slightly different amplification protocols were required for exon 12 (annealing temperature of 47°C) and for exon 9 (1 minute at 94°C, 90 seconds at 62°C and 2 minutes at 72°C). The same PCR amplification protocols were used in the second PCR round, to obtain the AC product, with the following modification: the PCR mixture contained 1 μl of the AB product, 40 pmol of primer A and 20 pmol of primer C (50 nucleotide universal GC-rich primer). The abundance and quality of the DNA fragments were analyzed by electrophoresis on 2% agarose gels, followed by ethidium bromide staining and inspection under UV.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was carried out according to Fischer and Lerman (27), as modified by Top et al. (28). GC-clamped amplified DNA fragments were run on 9% polyacrylamide gels containing

a linearly-increasing gradient of denaturant, ranging from 35 to 80%. Denaturing gradients were prepared from two stock solutions: (*i*) 9% acrylamide without denaturant (0%) and (*ii*) 9% acrylamide with 80% denaturant (100% denaturant is equivalent with 7 M urea and 40% (v/v) deionized formamide). The DGGE conditions for each exon were first calculated according to the melting temperature predicted by the melting maps; the optimal conditions, summarized in Table 2, were obtained when the DGGE bands appeared in the middle of the gel. Electrophoresis was performed at 80 V (6 $V.cm^{-1}$) for 20 hours at constant 60°C in TAE buffer (40 mM Tris-Acetate, pH 7.5, 1 mM EDTA). After electrophoresis, the gels were stained in ethidium bromide and examined under UV illumination.

Heteroduplex DNA fragments were artificially generated by mixing equal amounts of amplified normal and mutant DNA fragments. Samples were then denaturated at 100°C for two minutes and allowed to reanneal by cooling at room temperature.

Cloning and sequencing of PCR fragments

PCR fragments were ligated into the PT7 blue-vector according to the manufacturer's recommendations (PT7 blue cloning kit, Novagen, Madison, WI). Double stranded sequencing was performed using the ^{T7}Sequencing kit (Pharmacia, Uppsala, Sweden) with ^{35}S-αdATP (10 mCi/ml) (Amersham, Buckingham, U.K.) according to the protocol supplied by the manufacturer.

Results

Calculation of the melting maps

For amplification of each exon and flanking exon-intron junctions, primers were designed that were complementary to the intron sequences (6). To increase the sensitivity of DGGE, a 50-base-pair GC-clamp was included in either the 5'- or the 3'-end of each amplified fragment (22). A two-step PCR amplification protocol, schematically presented in Fig. 1, was used to incorporate the GC-clamp (26). For each exon, melting maps (24) of genomic fragments containing the GC-clamp either on the 5'-end or on the 3'-end were compared (Fig. 2). In each case, the GC-clamp attachment site that predicted the most uniform low-melting domain for the genomic sequence was chosen. The calculated melting maps, of which a representative one is shown in Fig. 2, indicate that in each one of the amplified 18 exons, the entire exon and its 5'- and 3'-flanking intron sequences are comprised in a uniformly low melting domain, thus predicting a high efficiency in screening for DNA sequence variations.

Screening for sequence variations

In Table 3 all sequence variations detected in the FH and the control population are listed systematically and given a serial designation according to the exon where they occur.

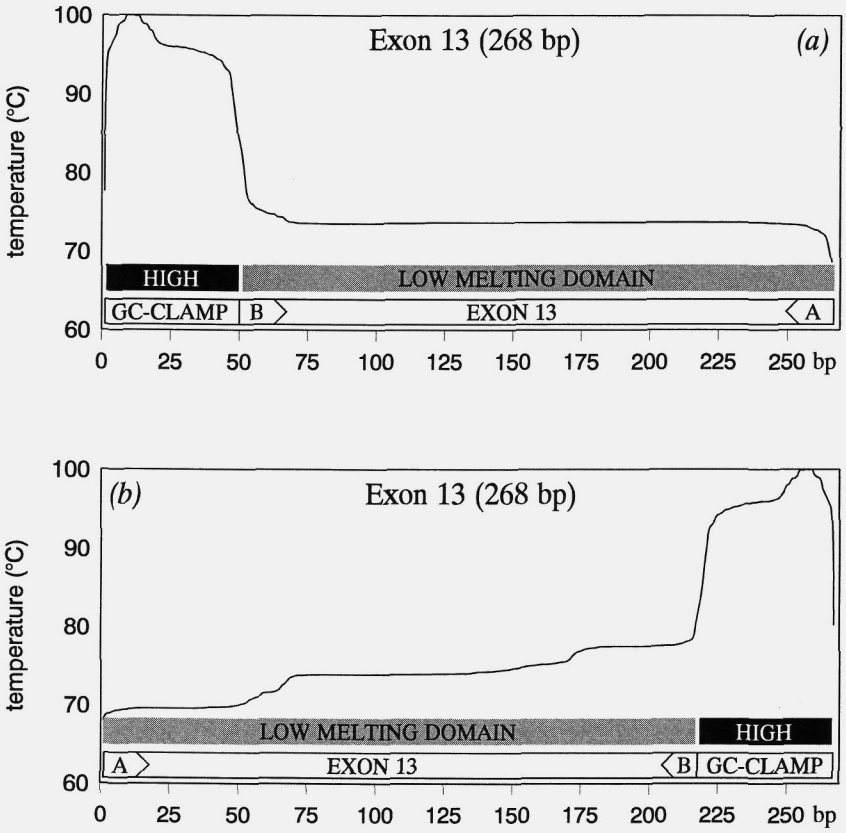

Figure 2. Calculated melting maps of exon 13 of the LDL receptor gene, carrying the GC clamp at either the 5'-end (a) or 3'-end (b), respectively. The melting temperature is plotted as a function of the nucleotide sequence. The size of the amplified fragment (including the GC-rich clamp) is indicated in parentheses. The configuration in panel (a), showing that the entire exon and its 5'- and 3'-flanking intron sequences are comprised in a uniformly low melting domain was the one selected.

Nucleotide alterations were revealed by the presence of two or more DNA bands on the denaturing gradient gels compared with a single band in normal samples. As shown in Fig. 3, many alterations were characterized by the typical "four-band" pattern for heterozygotes, consisting of two upper bands representing the heteroduplexes and two lower bands representing the homoduplexes of mutant and normal DNA strands, respectively.

Polymorphisms. Seven variants fulfilled criteria, which suggest that they represent polymorphisms, since they have been found both as homozygotes and heterozygotes in FH patients as well as in the control population (Table 3). To further test this hypothesis, the amplified fragments of the variant 8a, 12b and 13a in the FH population were analyzed by conventional restriction fragment length polymorphism (RFLP) analysis with the enzymes *Stu*I, *Hinc*II and *Ava*II, respectively. RFLP and DGGE analysis gave the very same results, thus demonstrating that these variants represent known common polymorphisms (15,18,19).

Table 3. Number of sequence variations.

Exon	Designation	Genotype					
		FH group			Control group		
		1.1[a]	1.2[a]	2.2[a]	1.1[a]	1.2[a]	2.2[a]
2	2a	31	1	0	32	0	0
	2b	0	6	26[b]	0	6	26[b]
3	3a	29	3	0	32	0	0
	3b	31	1	0	32	0	0
	3c	31	1	0	32	0	0
4	4a	31	1	0	32	0	0
	4b	31	1	0	32	0	0
	4c	31	1	0	32	0	0
	4d	31	1	0	32	0	0
	4e	31	1	0	32	0	0
	4f	31	1	0	32	0	0
6	6a	30	2	0	32	0	0
	6b	30	2	0	32	0	0
7	7a	29	3	0	n.t.[c]	n.t.	n.t.
8	8a	0	3	29[b,d]	0	2	30[b]
9	9a	31	1	0	n.t.	n.t.	n.t.
10	10a	31	1	0	32	0	0
	10b	30	2	0	32	0	0
	10c	10	20	2	10	17	5
11	11a	31	1	0	32	0	0
	11b	28	3	1	29	3	0
12	12a	29	3	0	32	0	0
	12b	8	14	10[d]	15	12	5
13	13a	10	15	7[d]	11	18	3
15	15a	31	1	0	32	0	0
	15b	19	13	0	20	10	2

[a] 1 and 2 indicate the absence or the presence of the sequence variation, respectively, as compared to the LDLR sequence published by Yamamoto et al. (29).

[b] presence of the sequence variation is more frequent than the absence (14,15).

[c] not tested.

[d] data representing the results of both DGGE and conventional RFLP analysis.

In case of the frequently occurring sequence variations 2b, 10c, 11b and 15b, the mutant fragments were cloned and sequenced. The mutations identified were fully compatible with those representing previously published neutral polymorphisms (14,16,17,20).

Candidate mutations. After DGGE analysis of all the 18 exons encompassing the LDL receptor protein coding and splice site consensus sequences in 32 heterozygous FH patients, we identified 28 unique mutations (Table 3), in addition to the common polymorphisms:

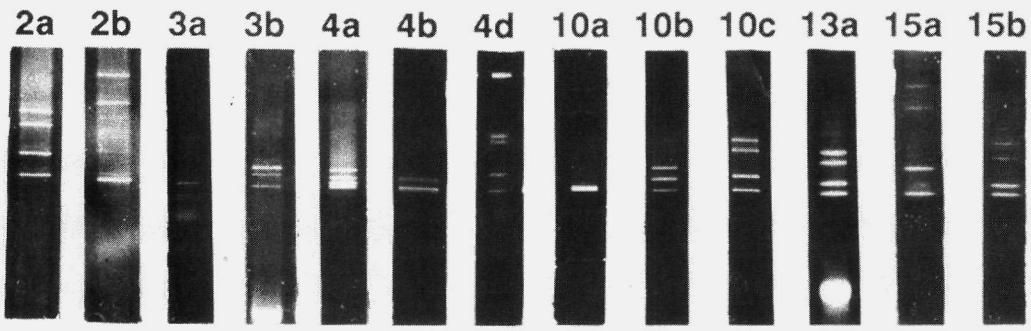

Figure 3. Ethidium bromide staining of gels revealing some of the different sequence variations found. The designation of the sequence variations is indicated.

these variants appear only in the heterozygous form, suggesting that they represent candidate FH-causing mutations. In order to further test this hypothesis 32 non-FH individuals were screened for the presence of aberrant patterns in exons 2, 3, 4, 6, 10, 11, 12 and 15. No aberrant DGGE patterns were found in this control group (Table 3).

In total, 26 out of 27 patients had a sequence change in only one allele, within either the coding region or the splice consensus sequences, which could not be attributed to polymorphisms, while one patient (FH-868) had two changes, in exon 3 (3a) and in exon 15 (15a), respectively, suggesting that FH-868 might be a compound heterozygote.

Identical banding patterns found in some patients suggest that the same mutation might be present in apparently unrelated patients. This hypothesis was further confirmed by mixing the corresponding PCR products, followed by heat-denaturation, reannealing at room temperature and DGGE. This reduces the number of different mutations found to 19. Among these, six different DGGE aberrant patterns were found in either two or three genetically unrelated patients; thirteen patterns appeared to be unique.

Among the twenty-eight mutations found, six were located in exon 4, five in exon 3, four in exon 6, three in exons 7, 10 and 12, and one in exons 2, 9, 11 and 15. In total, sixteen patients carried a mutation in exons 2-6, implying a high score in the ligand-binding domain of the LDLR. In exons 1, 5, 8, 13, 14, 16, 17 and 18 we did not find any abnormal DGGE pattern, except for exon 8 and 13, where the common *Stu*I and *Ava*II polymorphisms were detected, thus indicating that, at least in these two exons, the DGGE assay itself did not represent a limit for the detection of sequence changes.

Discussion

A considerable number of different mutations causing FH have been characterized during the past years. Part of these are large rearrangements, which can be easily detected by

conventional Southern blot analysis (3). In the vast majority of FH patients, mutations could not be identified by this method, suggesting that these are mainly point mutations or small deletions or insertions. The wide heterogeneity of the FH-causing mutations and the large size of the gene to be screened has retarded the characterization of point mutations (3). In addition, direct sequencing of PCR fragments of all exons of the LDL receptor gene is very laborious, and heterozygosity of mutations might cause equivocal interpretation. Therefore, a method that enables first to restrict the area (exon) of the gene to be sequenced is required. An early diagnosis of FH would allow, especially in children, a timely monitoring and treatment of this disorder. Moreover, it offers the possibility to study the role of mutations in the LDL receptor gene in mild hypercholesterolemia.

In the present study, sequence variations were detected in exon and splice site consensus sequences. DGGE does not allow for the exact identification of the variation observed: it only helps to define the region needed to be sequenced. Accordingly, an unequivocal discrimination between neutral polymorphisms and disease-causing mutations cannot always be made. However for seven variations, we obtained strong evidence that they represent common polymorphisms as they occur in both the heterozygous and homozygous form in the FH population as well as in the control population (Table 3). All other variations observed did not occur in the homozygous form and were not found at all in the control population, indicating that they represent candidates for FH-causing mutations.

Since we have not found any of these alterations in control individuals, we also exclude the possibility that the aberrant patterns observed are artefacts generated by the DGGE technique itself.

Among the 28 sequence variations found, exons 3 and 4 display the highest number of mutations, i.e. five and six, respectively. Considered all together, the alterations found in the exons encoding the ligand binding domain of the LDL receptor protein (exons 2-6) account for about 70% of the total number of mutations found. Two facts might explain this finding: (*i*) the FH patients included in our study displayed rather extreme LDL cholesterol levels (above 9.5 mmol/l). Therefore, they were expected to carry mutations that severely interfere with the functionality of the LDL receptor. Mutations occurring in the ligand binding domain are likely to produce a severely defective receptor protein. (*ii*) The CpG dinucleotide has been indicated as a "hot spot" for mutations (30) and indeed half of the point mutations described in the LDL receptor gene involved the CpG dinucleotide. Considering the high CpG content of exon 4 and the fact that this exon codes for a critical region of the binding domain of the LDL receptor, Gudnason et al. (31) suggested that exon 4 might be the site where mutations most frequently occur. Our results support this statement.

The pattern found for the sequence variation type 9a (Table 3) appeared to be identical to the common exon 9 mutation found in the South Afrikaan FH population (a G to A transition at codon 408) (7). This identity could easily be proven by mixing the exon 9 PCR products of the patient carrying the sequence variation type 9a with the one of a patient carrying the known South Afrikaan mutation, followed by heat-denaturation, reannealing at room temperature and DGGE. Similar banding patterns were obtained with or without

mixing. Thus, the DGGE assay offers an additional advantage: since each sequence alteration produces a distinctive, unique banding pattern, DGGE analysis enables to compare, within the same PCR fragment, patterns of newly identified sequence alterations with that of known mutations. Using this test, we observed that some mutations occur in more than one patient. The subjects under investigation have been recruited among the patients attending the Lipid Clinic of the Leiden University Hospital, where a certain genetic homogeneity can be expected. The finding that six different mutations have been found in at least two patients suggests that, indeed, some specific mutations might be fairly frequent in the Dutch population (10). Although all subjects were apparently unrelated, a deeper genealogic investigation might reveal the common ancestry of some patients.

In 5 out of the 32 heterozygous FH patients included in this study we did not identify any sequence abnormality, except well-characterized polymorphisms. The reason for this might be: (*i*) lack of full resolution power of DGGE itself. It cannot be excluded that certain sequence changes, due to their peculiar location in the fragment, might escape detection by this technique, although the melting maps of each fragment predicted that almost all, if not all, sequence variations present should be identifiable: (*ii*) the mutations might be in regions of the LDL receptor gene that have not been analyzed in the present study, such as the promoter region and most of the 3'-untranslated region of exon 18. However, previous studies have shown that mutations in the promoter region (from position -512 to -66) of the LDL receptor are either absent (32) or at least very rare (3). Specific trinucleotides, present in the 3'-untranslated region of the 7α-hydroxylase and avian apolipoprotein II genes, have been found to be associated with mRNA stability (33,34). Similarly, mutations involving these sequences occurring in the 3'-untranslated region of the LDL receptor gene might also give an origin to mRNA deficiency. Moreover, novel important regulatory sequences, even in an intron, further away from the LDL receptor gene cannot be excluded.

The involvement of genes in the expression of FH other than the LDL receptor gene, must also be considered. Patients carrying the gene for familial defective apoB were excluded from this study. However, abnormal lipoprotein profiles can be produced by more than one defective gene and the presence of overlapping clinical symptoms, like the presence of tendon xanthomas and/or a positive family history of coronary heart disease, might lead to an erroneous diagnosis of FH.

Sequence analysis of the mutant fragments is ongoing; this will definitively prove the causal relationship between the aberrant DGGE pattern and the disease. It will also reveal how many of the different sequence changes identified in this study represent mutations described before (3). By DGGE we have screened not only the coding sequence but also the splice site sequences at intron/exon junctions. At present, only very few FH-causing mutations occurring at splice sites have been described (3, 28 and P. Lombardi et al., manuscript submitted), possibly due to the fact that, so far, primers exactly flanking the exons were used for PCR amplification of the fragments to be screened.

In conclusion, in this study we have shown that DGGE is a reliable and rapid screening method for the detection of the presence of sequence variations in the LDL receptor gene.

DGGE facilitates the characterization of the FH-causing mutations by restricting the region of the gene that requires subsequent sequence analysis. In this respect, the knowledge of the molecular defect, producing the aberrant DGGE pattern, would allow to lay the basis for an inventory of the mutations present in the Dutch FH population and, subsequently, to develop rapid, PCR-based techniques, for the identification of specific mutations.

Considering the high capacity and sensitivity, this method might also contribute to elucidate the role of LDL receptor mutations in mild hypercholesterolemia.

Acknowledgements

This study was financially supported by the Netherlands Heart Foundation (P.L.: project # 89.057 and A.v.d.M.: project # 88.086).

We wish to acknowledge Dr. L.S. Lerman for providing the computer program MELT 87. Mrs. Inge de Bruin is thanked for excellent technical assistance. Dr. Bert Top is acknowledged for helpful discussions. We are grateful to Drs. J. Defesche and J.J.P. Kastelein, Amsterdam Medical Center, for performing the analysis of the B3500 variant (FDB).

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Chapter 10

SUMMARY AND DISCUSSION

The studies presented in this thesis deal with different biochemical and genetic aspects of the LDL receptor function and its involvement in cellular cholesterol metabolism. Rapidly proliferating cells, such as tumour cells, require cholesterol for incorporation into membranes. Cholesterol can be supplied either by endogenous synthesis or by uptake of LDL as mediated by the LDL receptor pathway. Indeed, several reports have shown that *in vitro* tumour cells avidly assimilate LDL via the LDL receptor. *In vivo* data (Norata et al., 1984) tend to confirm these observations, indicating that *in vivo* tumour cells might also depend on LDL for their proliferation. These preliminary studies suggested the possibility of using LDL as a vehicle for specific targeting of cytotoxic drugs to tumour cells (Gal et al., 1981; Catapano, 1987). To further explore this possibility, *in vivo* studies were performed with seven experimental murine tumours, in which the uptake of LDL was measured and the properties of the LDL receptor as expressed by the tumour cells were compared to those of the hepatic receptor (Chapter 2). Four out of seven tumours tested showed a higher relative uptake of LDL as compared to the liver; in one (M5) the uptake by the tumour and liver was similar, while two displayed a relatively low LDL uptake. Similar results were obtained when the binding of LDL to membranes, prepared from the tumour tissues, was measured, suggesting that the expression of the LDL receptor on the tumour cell membranes is related to the *in vivo* uptake of LDL. Furthermore, the receptor in tumour cells was found to be similar, if not identical, to the hepatic receptor. We also considered whether differences in the rate of uptake and degradation of LDL by the tumour tissues as compared to other organs might influence the targeting efficiency. Experiments performed using [^{14}C]sucrose-labelled LDL are in agreement with those in which ^{125}I-labelled LDL were used, indicating that a high expression of the LDL-receptor on tumour cells correlates with a higher uptake of LDL by the tumour. The correlation between the high *in vitro* expression of LDL receptors and the high *in vivo* uptake suggests that *in vitro* experiments are predictive of the *in vivo* situation and could be therefore used for discriminating tumours in which the delivery of drugs via the LDL receptor pathway might be effective.

Cultured human skin fibroblasts have been the most widely used cell line for studying LDL receptor abnormalities with regard to the receptor function, synthesis and post-translational processing. Two practical reasons limit their diagnostic application: (*i*) they can only be obtained from skin biopsies and (*ii*) since they grow in a monolayer, they do not

represent an abundant source of biological material from which the receptor can be easily extracted. Lymphocytes, obtained from human blood, can be transformed by Epstein-Barr virus (EBV-L) into rapidly proliferating cells, which might offer an alternative to fibroblasts in the study of LDL receptor abnormalities in FH patients (Chapter 3). The properties of the LDL receptor in this cell line were studied and binding studies revealed the presence of high-affinity binding sites for LDL in EBV-L. These sites are specific for LDL since they are not expressed in EBV-L obtained from a homozygous FH patient. The LDL receptor in EBV-L was found to share many of its properties with the fibroblast receptor: it is sensitive to EDTA, it is up- or down-regulated respectively in the absence or presence of LDL in the medium, and on ligand blotting, it shows the same apparent molecular weight of the receptor protein. These results indicate that EBV-L are a suitable model system for biological and biochemical studies on the LDL receptor protein. In this respect, our approach is applied in two recently published reports, in which LDL receptor activity was measured in EBV-L as a diagnostic test for the receptor deficiency (Delattre et al., 1991) or as a complementary approach to RFLP analysis in order to study the cosegregation of the defective allele in a family with FH (Humphries et al., 1993).

Another cell line which has traditionally been used to study lipoprotein metabolism, as a model for the human hepatocytes, is the human hepatoma cell line HepG2. Considering that the liver is the organ which mainly contributes to LDL catabolism, it is conceivable that differences in the function and regulation of the hepatic receptor can have a major impact in the overall cellular cholesterol metabolism *in vivo*. As we have seen in EBV-L, one of the major features of the LDL receptor activity is the feedback regulation. In this respect, previous studies have shown that the LDL receptors both in HepG2 and freshly isolated human hepatocytes are much less responsive to down-regulation by extracellular LDL than the LDL receptors in fibroblasts (Havekes et al., 1986a,b). One possible explanation is that in HepG2 cells both the LDL cholesterol and the endogenously synthesized cholesterol are directed to a cholesterol ester pool or to extracellular cholesterol acceptors, like heavy HDL, rather than to the cholesterol pool involved in the regulation of the LDL receptor activity (Havekes et al., 1987). We reasoned that more information about the intracellular pathway of LDL might help in explaining the difference between fibroblasts and HepG2 cells in their ability to modulate LDL-receptor activity in response to extracellular LDL cholesterol (Chapter 4). Pulse-chase studies indicated that upon incubation of HepG2 cells with ^{125}I-LDL the appearance of degradation products started at 90 minutes, whereas in fibroblasts this lag time was only 30 minutes. In HepG2 cells LDL is internalized at the same rate as in fibroblasts. To study the intracellular traffic of LDL in the two cell lines, we used Percoll gradient subcellular fractionation, which allows for the separation of subcellular compartments according to differences in their buoyant densities, i.e. the early endosomes from the lysosomes. As has been recently described (Stoorvogel et al., 1991), the late endosomes originate from the early endosomes through a maturation process. During this process their density increases to that of the lysosomes. However, both in fibroblasts and HepG2 cells LDL is equally rapidly shifted from the endosomal to the late-endosomal plus

lysosomal compartment, indicating that in HepG2 cells: (*i*) either the fusion process between the late endosomes and the lysosomes is impaired or (*ii*) the degradation of LDL itself in the lysosomes is less efficient than in fibroblasts. From these studies we concluded that a low degradation efficiency of LDL in HepG2 cells may contribute to explain the relatively weak down-regulation of the LDL receptor activity upon incubation of cells with LDL.

In HepG2 cells, VLDL and lipoprotein lipase treated VLDL (LPL-VLDL), the latter representing VLDL-remnants, are poorly degraded as compared to LDL (Chapter 5). Studies on the differential subcellular distribution of these particles show that the transport of LPL-VLDL and VLDL to the late endosomal-lysosomal compartment is severely retarded as compared to LDL. However, once present in the lysosomes, LPL-VLDL and VLDL are more rapidly degraded than LDL. We found that the rate of retroendocytosis of LPL-VLDL and VLDL is higher than that of LDL (up to 20%, 40% and 10%, respectively). This indicates that a substantial amount of VLDL and LPL-VLDL once taken up is diverted from the routing to the lysosomes and excreted as intact particles into the medium. As an explanation for the increased rate of retroendocytosis of LPL-VLDL and VLDL, we propose that, due to the slower transport to the lysosomes, these particles reside for a longer time than LDL in the early endosomal compartment, thus increasing the probability that they return to the cell surface by retroendocytosis. Therefore, both a slower transport to the lysosomal compartment and a higher rate of retroendocytosis, possibly as the consequence of the longer residence-time in the early endosomes, are responsible for the poor degradation of VLDL and LPL-VLDL by HepG2 cells.

ApoE is known to be the major determinant for regulating the metabolic fate of VLDL (Mahley, 1988) and LPL-VLDL (Mulder et al., 1991). Recent studies by Tabas et al. (1991) have shown that the multivalent binding of β-VLDL through apoE to the LDL receptor leads to a divergent endocytotic pathway as compared to LDL. Following the same line of reasoning, we suggest that the high-affinity, apoE mediated binding of VLDL and LPL-VLDL to the LDL receptor might be responsible for the retarded intracellular routing of these particles, possibly due to its greater resistance to the acid-mediated release of the ligand from the receptor.

A low degradation efficiency of VLDL and LPL-VLDL would also imply a relatively poor down-regulation of the LDL receptor activity upon incubation of cells with these lipoproteins as compared to LDL. Epidemiological studies suggest that the down-regulation of the LDL receptor activity in the liver by VLDL and VLDL-remnants depends, at least partly, on the polymorphism of apoE through affecting the binding of these lipoproteins to the receptor. Whether or not the apoE polymorphism also interferes with the efficiency of cellular degradation of VLDL or VLDL-remnants (as a consequence of a retarded intracellular transport to the lysosomal compartment) is currently under investigation.

LPL is the enzyme that mediates the conversion of VLDL into VLDL-remnants. Recently, LPL has been assigned a second function: LPL dramatically increases the cellular binding not only of apoE-containing lipoproteins (Beisiegel et al., 1991) but also of LDL (Mulder et al., 1992). Studies in HepG2 cells, fibroblasts and LDL receptor-deficient

fibroblasts (Mulder et al., 1992) have ruled out the possible involvement of the LDL receptor and of the LDL receptor related protein (LRP) in the LPL-mediated stimulation of the binding. The finding that the LPL-mediated binding of lipoproteins is inhibited by pre-treatment of the cells with heparinase led to the conclusion that the major part of the LPL-stimulated binding of LDL and VLDL is caused by a LPL-mediated bridging between heparan sulphate proteoglycans (HSPG) on the plasma membrane and the lipoproteins. In Chapter 6, we report that the major part of the (LPL-mediated) HSPG-bound lipoproteins are taken up via the LDL receptor, while only a small amount of the LPL-mediated binding of LDL is internalized together with HSPG and independently of the LDL receptor. Therefore, the mechanism for LPL-mediated binding and uptake of LDL includes binding on the plasma membrane, after bridging to HSPG, with the transfer of the major part of the LDL to the LDL receptor. Thereafter, the HSPG-bound LDL and VLDL are rapidly internalized via the LDL receptor-recycling system.

Several studies have demonstrated that the LDL receptor activity determines the plasma lipid concentrations (Brown and Goldstein, 1986). Environmental and genetic factors that alter the LDL receptor activity or its ligands often lead to an accumulation in the plasma of specific lipoproteins and, consequently, to hyperlipidemia. In this thesis we have focused on one of the genetic lipoprotein disorders that have been reported: familial hypercholesterolemia (FH).

FH is a genetic disorder of lipid metabolism, characterized by elevated plasma LDL cholesterol levels and by an increased risk for developing premature atherosclerosis. It is caused by mutations in the LDL receptor gene, leading to a varying degree of defects in the receptor protein function, depending on whether the synthesis, post-translational processing, ligand-binding activity, internalization or receptor-recycling is affected.

About 150 different mutations have been identified so far (Hobbs et al., 1992). Detection of large rearrangements (deletions/insertions) is relatively easy and can be done by conventional techniques like Southern blotting. Unfortunately, in the LDL receptor gene large rearrangements represent only about 5% of all the mutations, whereas the majority of mutations causing FH are point mutations or small deletions/insertions that can only be detected after screening of the entire gene. Despite the high number of mutations which have already been described (Hobbs et al., 1992), the characterization of additional ones still represents a major goal, and will provide further insight in the structure-function relationship of the receptor protein. Furthermore, the knowledge of the molecular defect underlying a mutation allows for the setting up of an easy PCR-based technique which can be used for rapid screening of specific mutations in the FH population and for individual FH diagnosis.

In this thesis we describe different approaches used for the identification and characterization of mutations in the LDL receptor gene.

As a strategy for screening for point mutations or small deletions/insertions in the LDL receptor gene, we applied denaturing gradient gel electrophoresis (DGGE), which in recent years has proved to be a powerful method for almost complete detection of the presence of disease-related mutations and polymorphisms (Kogan and Gitschier, 1990). In Chapter 7 the

characterization of a mutation in the LDL receptor gene of a true homozygous FH patient is described. The combined use of DGGE and DNA sequence analysis revealed a unique A to G transition in the penultimate 3'-nucleotide of intron 16 of the LDL receptor gene, which disrupts the acceptor splice site. cDNA sequence analysis indicated that a cryptic splice site was activated in intron 16, upstream from the original splice site, leading to the inclusion of 62 nucleotides and a reading frame-shift. The resulting new translation product contains a stretch of 154 amino acids at the C-terminal that have no resemblance to the normal receptor protein. To elucidate the biological effects of the mutation, the structural and functional properties of the mutated LDL receptor protein were studied. Immunoprecipitation of the newly synthesized receptors showed that an aberrant precursor form of the LDL receptor protein was synthesized, approximately 10 kD larger than normal, which is not further processed to a mature form. Some 50% of the normal LDL binding activity was found on the cell surface of the patient's fibroblasts, whereas internalization and degradation of LDL were totally abolished. A few (5 in total) splice site mutations have been reported to occur in the LDL receptor gene, while several splice mutations have been found in other genes, leading to the activation of cryptic splice sites and/or inclusion of the unspliced intron and, ultimately, resulting in a reading frame-shift. In our case the new protein reading-frame enabled us to predict an elongation of about 90 amino acids in the LDL receptor protein. This elongation was consistent with the 10 kD higher apparent molecular weight of the mutated receptor protein on SDS gel electrophoresis. The mutation affects two of the functional domains of the LDL receptor protein: the membrane spanning domain (exon 16 and 5'-end of exon 17) and the cytoplasmic domain (3'-end of exon 17 and 5'-end of exon 18). Although the mutant protein appears to be incorrectly glycosylated and its conformation disrupted, due to the frame-shift down-stream exon 16, the mutant receptors are transported to the plasma membrane, as indicated by the presence of lipoprotein-binding activity on the cell surface. Since the surface binding was not followed by internalization, we classified this mutation as internalization-defective. We suggest that the 8 amino acid residues, forming the first part of the transmembrane domain, which are intact in the mutant protein are sufficient to anchor the mutant receptor to the cell membrane. However, the disruption of the entire cytoplasmic domain and the unusually long C-terminal amino acid sequence, which has no resemblance to the normal LDL receptor, abolishes the signal for clustering in coated pits, thereby preventing the internalization of the ligand-receptor complex.

Allele-specific transcript PCR (AST-PCR), a recently developed method to study allele-specific transcripts (Top et al., 1991), was applied to study the prevalence of mRNA deficient alleles of the LDL receptor gene in heterozygous FH patients (Chapter 8). Allele-specific mRNA deficiency was observed in 12 out of 48 patients. Haplotype analysis suggested that a limited number of haplotypes may be associated with the mRNA deficiency. In one family co-segregation between a mRNA deficient LDL receptor allele and FH was demonstrated. In one patient, DGGE screening of the entire coding sequence in combination with sequence analysis revealed a single base insertion in exon 11, which produces a frame-shift and a premature stop codon. Other reports have demonstrated that mutations which

introduce a premature termination of translation are associated with decreased intracellular levels of the mutant mRNA, suggesting that the mutation we have found in exon 11 is likely to be responsible for the mRNA deficiency. However, the exact mechanism by which mRNA abundance is affected by premature termination codons is not known. DGGE analysis also revealed the location of the mutation in several other patients and sequence analysis is currently ongoing. The characterization of the molecular defect in mRNA deficient alleles will help in answering the question as to whether specific mutations are associated with mRNA deficiency and, possibly, will provide more information about the mechanism underlying mRNA deficiency.

To optimize the efficacy of the therapeutic intervention in heterozygous FH, an early diagnosis of the disease is required. In case of heterozygous FH an unequivocal diagnosis cannot always be made, especially in children, in whom the clinical signs of the disease are not apparent yet, or in adults who did not develop severe hypercholesterolemia, xanthomas or CAD. In these cases a rapid laboratory test, capable of detecting the molecular defect responsible for the disease, would provide a much more accurate and reliable tool for the individual diagnosis of FH.

In Chapter 9 we describe the development of a DGGE assay which enables one to efficiently screen the coding and splice consensus sequences of the LDL receptor gene for the presence of sequence variations. In a group of 32 apparently unrelated heterozygous FH patients, we identified the previously reported common polymorphisms and twenty-eight sequence variations. Among these, six have been found in more than one patient, suggesting that some specific mutations might be rather frequent in the Dutch population. Since all the sequence variations identified were unique and none of them has been found in non-FH individuals, we strongly suggest that these are most likely to be FH-causing mutations. An additional major advantage offered by DGGE is that each mutation produces a distinctive banding pattern. This allows one to compare newly identified sequence variations with known mutations and therefore, to identify mutations solely on the basis of their DGGE pattern. Indeed, among the sequence variation identified, one appeared to be identical to a common mutation found in the South Afrikaner population. Although DGGE does not reveal the nature of the sequence alteration, it facilitates the characterization of FH-causing mutations by restricting the region of the gene which requires sequence analysis. Sequence analysis of the mutant fragments is ongoing; this will definitively prove the causal relationship between the aberrant DGGE pattern and the disease. It will also reveal how many of the different sequence changes identified in this study represent mutations described before. The knowledge of the molecular defect causing the aberrant DGGE pattern will, ultimately, allow to generate a comprehensive overview of the mutations present in the Dutch population and, subsequently, to develop rapid PCR-based techniques, properly tailored according to the nature of the mutation, for the identification of specific mutations in a larger population sample. If, as it has been suggested before (Jeenah et al., 1993), different mutations are associated with different clinical consequences or require different medical treatment, the identification of the molecular defect underlying FH may be even more beneficial for the

individual patient (Gudnason et al., 1993). We would like to conclude that DGGE provides an excellent tool for the detection of disease-causing mutations in the LDL receptor gene.

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SAMENVATTING

In dit proefschrift wordt biochemisch en genetisch onderzoek beschreven waarbij de structuur en functie van de lage dichtheid lipoproteïne (LDL)-receptor in het intra-cellulaire cholesterolmetabolisme centraal staat. Eerder onderzoek wees op de mogelijkheid om het LDL-deeltje te gebruiken om cel-dodende stoffen naar tumorcellen te transporteren. De relevantie van dit model werd bij 7 verschillende muize-tumoren onderzocht. In hoofdstuk 2 wordt de expressie van de LDL-receptor in deze verschillende tumorcellen vergeleken met die van de lever van de muis. Vier van de 7 tumoren bleken een relatief hoge LDL-opname te vertonen. In één van deze tumoren (M5) was de opname vergelijkbaar met die van levercellen, terwijl de overige twee tumoren een relatief lage opname van LDL vertoonden. Vergelijkbare resultaten werden verkregen wanneer de binding van LDL aan geïsoleerde celmembranen van deze tumoren werd geanalyseerd. De expressie van de LDL-receptor in deze tumoren *in vitro* bleek overeen te komen met de *in vivo* LDL-opname. Tevens vertoont de receptor in de tumorcel identieke eigenschappen als de receptor in de lever. De sterke correlatie tussen de expressie van de LDL-receptor *in vitro* enerzijds en de *in vivo* LDL-opname anderzijds, betekent dat de *in vitro* experimenten de *in vivo* situatie weerspiegelen, en dus gebruikt kunnen worden om onderscheid te maken tussen tumoren welke wel, en tumoren welke niet in aanmerking komen om effectief te worden bestreden met cel-dodende stoffen via LDL-receptor afhankelijke opname.

De gekweekte menselijke huidfibroblast is tot heden de meest gebruikte cel om LDL-receptor-afwijkingen te bestuderen. Dit celtype wordt gebruikt ter bestudering van de LDL-receptor functie, de synthese van de receptor en de posttranslationele regulatie. Er zijn echter twee redenen die de fibroblast minder geschikt maken om de primaire eiwit-structuur van de LDL-receptor te bestuderen bij mensen: (*i*) fibroblasten kunnen alleen met behulp van een huidbioptie worden verkregen, en (*ii*) er kunnen slechts moeizaam voldoende cellen worden gekweekt om voldoende LDL-receptoren te isoleren voor eiwit-structuur-onderzoek. Zogenaamde Epstein-Barr virus getransformeerde lymfocyten (EBV-L) vormen daarentegen een welhaast onuitputtelijke en gemakkelijk verkrijgbare bron van snel groeiende cellen. Dit type cel zou daarom een aantrekkelijk alternatief kunnen zijn voor de fibroblast ter bestudering van afwijkingen in de LDL-receptor bij patiënten met familiaire hypercholesterolemie (FH). De geschiktheid van de EBV-L cellen is getest met bindingsstudies waarbij bleek dat er hoog-affiniteit bindingsplaatsen voor LDL op deze cellen voorkomen (hoofdstuk 3). Directe vergelijking met EBV-L afkomstig van een patiënt welke homozygoot was voor een LDL-receptor defect, toont aan dat deze bindingsplaatsen afwezig zijn. De LDL-receptor in de EBV-L blijkt vergelijkbaar met de LDL-receptor op fibroblasten: de receptor is gevoelig voor EDTA, de regulatie is afhankelijk van de aanwezigheid van LDL in het kweekmedium en uit ligand-blottingsexperimenten blijkt dat

de twee receptoren een vergelijkbaar molecuulgewicht hebben. Geconcludeerd kan worden dat EBV-L een aantrekkelijk alternatief zijn voor het gebruik van fibroblasten bij het bestuderen van het LDL-receptor polymorfisme bij FH patiënten.

De menselijke lever-cellijn HepG2 is de meest gebruikte cellijn als model om de functie van de LDL-receptor in de levercel te bestuderen. Aangezien de lever *in vivo* het belangrijkste orgaan is voor de LDL-afbraak, zijn studies naar de functie en regulatie van de lever-receptoren erg belangrijk voor het totale *in vivo* cholesterolmetabolisme. Zoals reeds beschreven voor de EBV-L, is een van de belangrijkste eigenschappen van de LDL-receptor de zogenaamde "feed-back" regulatie. Uit eerdere studies kan geconcludeerd worden dat zowel HepG2-cellen als vers geïsoleerde levercellen minder gevoelig zijn voor een onderdrukking van de receptor-synthese door LDL dan fibroblasten. Het mechanisme van het verschil in regulatie van de LDL-receptor in HepG2 cellen en fibroblasten werd met cel-biologische technieken bestudeerd (hoofdstuk 4). Hoewel de LDL-opname en intra-cellulair transport naar het laat-endosomaal/lysosomaal compartiment in HepG2 cellen vergelijkbaar is met dat in fibroblasten, is de LDL-afbraak zeer inefficiënt en sterk vertraagd in de HepG2 cellijn. Twee mogelijke mechanismen voor deze bevindingen worden gesuggereerd: (*i*) de samensmelting tussen de laat-endosomen en de lysosomen is verstoord, en (*ii*) de afbraak van LDL in de lysosomen is minder efficiënt. De minder efficiënte degradatie van LDL in HepG2 cellen is mogelijk mede oorzaak voor de geringe onderdrukking van de synthese van LDL-receptoren door LDL.

Ook zeer lage dichtheid lipoproteïnen (VLDL) en lipoproteïne lipase-behandeld VLDL (LPL-VLDL; als alternatief voor VLDL-remnants) worden, indien eenmaal opgenomen via de LDL-receptor, slecht afgebroken door HepG2 cellen (hoofdstuk 5). Uit studies naar de sub-cellulaire verdeling van deze lipoproteïnen bleek dat het transport van VLDL en LPL-VLDL naar het laat-endosomaal/lysosomaal compartiment sterk vertraagd is ten opzichte van LDL. Echter, eenmaal in de lysosomen worden VLDL en LPL-VLDL sneller afgebroken dan LDL. Ook is de retro-endocytose van VLDL en LPL-VLDL hoger dan die van LDL. Een mogelijke verklaring hiervoor is dat deze deeltjes langer dan LDL in het vroeg-endosomaal compartiment verblijven, waardoor de kans op transport terug naar het celoppervlak vergroot wordt. Geconcludeerd kan worden dat het transport naar de lysosomen en een sterkere retro-endocytose verantwoordelijk zijn voor de verminderde afbraak van VLDL en LPL-VLDL door HepG2 cellen. De oorzaak van dit vertraagde intra-cellulaire transport zou kunnen liggen in een verminderde zuur-gemedieerde intra-cellulaire ontkoppeling van de ligand en van de receptor, als gevolg van de apoE-afhankelijke binding van VLDL en LPL-VLDL. Bij de binding van LDL aan de receptor zorgt apoB voor de binging, hetgeen minder sterk is dan die waarbij apoE de ligand is.

Het is bekend dat lipolyse van chylomicronen door lipoproteïne lipase (LPL) en VLDL de binding aan levercel-receptoren verbetert. Er is echter gesuggereerd dat ook LPL zèlf de binding van VLDL en LDL aan HepG2 cellen en fibroblasten zeer sterk stimuleert (tot 100 maal). In hoofdstuk 6 wordt aangetoond dat een groot deel van de LPL-gemedieerde binding van LDL en VLDL niet via lipoproteïne-receptoren verloopt, maar wordt veroorzaakt door

een LPL-gemedieerde binding van deze deeltjes aan heparan sulfaat proteoglycanen (HSPG), welke op de celmembranen aanwezig zijn. LPL functioneert dus als een soort brug tussen enerzijds de lipoproteïnen in het medium en anderzijds het HSPG op de plasmamembraan. Zeer opmerkelijk is dat een groot deel van de LPL-gemedieerde HSPG-gebonden deeltjes vervolgens wèl via de LDL-receptor wordt geïnternaliseerd, terwijl slechts een gering gedeelte via andere (langzame) routes door de cel wordt opgenomen.

Algemeen wordt aangenomen dat de plasma LDL-cholesterol concentratie door de LDL-receptor activiteit wordt gereguleerd. Omgevings- en genetische factoren welke in staat zijn om de LDL-receptor activiteit te beïnvloeden, zijn daarom mede verantwoordelijk voor een verhoging in het plasma van het gehalte aan LDL-cholesterol (hypercholesterolemie). In dit proefschrift wordt veel aandacht geschonken aan familiaire hypercholesterolemie (FH). FH is een genetisch bepaalde afwijking in het lipoproteïne metabolisme, die gekarakteriseerd wordt door een sterk verhoogde LDL-cholesterol concentratie in het plasma en door een sterk verhoogd risico voor vervroegde atherosclerose. FH wordt veroorzaakt door mutaties in het gen voor de LDL-receptor welke op verschillende manieren het goed functioneren van het LDL-receptor eiwit kunnen beïnvloeden. Zo zijn storingen beschreven in de aanmaak van het eiwit, in de posttranslationele processing, in de ligand-binding, in de opname van de receptor door de cel en in de recirculatie van de receptor. Er zijn tot nu toe minstens 150 verschillende LDL-receptor mutaties beschreven. Ondanks dit grote aantal reeds beschreven mutanten, blijft de karakterisatie van nog onbeschreven varianten van groot belang om uiteindelijk een eenvoudige moleculair-genetische methode te kunnen ontwikkelen voor individuele patiënten. In de hoofdstukken 7-9 worden verschillende manieren ter bestudering en karakterisering van LDL-receptor mutanten beschreven.

In hoofdstuk 7 wordt de mutatie in het LDL-receptor gen van een homozygote FH patiënt beschreven. Door een gecombineerde toepassing van "denaturerende gradient gel electroforese" (DGGE) en DNA sequentie-analyse werd een A → G transitie van de op één na laatste base van intron 16 van het LDL receptor (LDLR) gen gevonden. Deze transitie verstoort het proces van "splicing", en resulteert uiteindelijk in een langer eiwit waarvan de laatste 154 aminozuren sterk afwijken van het normale LDL-receptor eiwit. Om de invloed van deze verandering in primaire structuur van het eiwit nader te bestuderen, werd een aantal aanvullende experimenten verricht. Uit immunoprecipitatie van nieuw aangemaakt LDL-receptor eiwit bleek de precursor-vorm 10 kD langer te zijn dan normaal. Ook bleek dat deze langere precursor niet verder kon worden omgezet. Fibroblasten van deze patiënt bleken in staat om slechts 50% van de normale hoeveelheid LDL te kunnen binden. Dit gebonden LDL kan echter niet door de cel worden opgenomen en afgebroken.

Een recent ontwikkelde techniek voor het aantonen van allel-specifieke transcripten met behulp van PCR (AST-PCR) werd gebruikt om de heterozygotie voor mRNA-deficiënte LDL-receptor allelen in heterozygote *FH* patiënten te bestuderen (hoofdstuk 8). Wij waren in staat om bij 12 van de in totaal 48 (25%) onderzochte FH patiënten, allel-specifieke mRNA-deficiëntie aan te tonen. Uit haplotypering van deze patiënten kon worden afgeleid dat slechts een beperkt aantal haplotypes associeert met mRNA-deficiëntie. Na DGGE- en

DNA sequentie-analyse van het gehele LDLR gen van een patiënt bleek een insertie van één base, resulterend in een vervroegde stop-codon, de oorzaak van de mRNA-deficiëntie te zijn.

Een zo vroeg mogelijke herkenning van FH is zeer belangrijk. Dit maakt effectieve behandeling van deze ziekte mogelijk. Vooral bij kinderen, welke de ziekte nog niet openbaren, en bij volwassenen welke geen ernstige hypercholesterolemie, xanthomen of CAD hebben, is een juiste diagnose van FH niet altijd mogelijk. In dit soort gevallen kan een snelle en diagnostisch betrouwbare test uitkomst bieden. In hoofdstuk 9 beschrijven wij de ontwikkeling van een DGGE methode waarmee op de aanwezigheid van een mutatie in alle coderende en belangrijke aangrenzende gedeelten van het LDLR gen kan worden gescreened. Met deze methode vonden wij bij 28 van de 32 niet verwante heterozygote FH patiënten, naast reeds beschreven polymorfismen, een mutatie in het gen. Zes van deze mutaties werden bij meer dan één patiënt gevonden, hetgeen ons doet veronderstellen dat deze mutaties relatief frequent voorkomen in de Nederlandse FH populatie.

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ABBREVIATIONS

ACAT	acyl-CoA:cholesterol acyltransferase
apo	apolipoprotein
AST-PCR	allele specific transcript-polymerase chain reaction
BSA	bovine serum albumin
β-VLDL	β-migrating very low density lipoprotein
d	density (g/ml)
DGGE	denaturing gradient gel electrophoresis
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
FH	familial hypercholesterolemia
HDL	high density lipoprotein
HSA	human serum albumin
HSPG	heparan sulphate proteoglycan
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LPL	lipoprotein lipase
LPL-VLDL	lipoprotein lipase-treated very low density lipoprotein
LRP	low density lipoprotein receptor-related protein
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulphate
TC	total cholesterol
TCA	trichloroacetic acid
TG	triglyceride
VLDL	very low density lipoprotein

CURRICULUM VITAE

The author of this thesis was born in Milan on April 4th, 1960. From 1974 to 1979 she attended the high school at "Ginnasio-Liceo A. Manzoni", Milan. In 1979 she started a University degree course in Pharmaceutical Chemistry and Technology, School of Pharmacy, at the State University of Milan. In 1984 she joined the Laboratory of Pharmacology of Lipid Transport and Metabolism (led by Prof. Dr. A.L. Catapano), Institute of Pharmacological Science (director: Prof. Dr. R. Paoletti), University of Milan. In 1986, after the completion of an experimental thesis on the subject of the expression of the low density lipoprotein receptor in experimental tumours in mice, she was awarded her Master Degree. From 1986 to 1987 she worked at the above institute as a postgraduate fellow.

In 1987 she married and came to The Netherlands. In February 1988, she started working at the Gaubius Institute, TNO (headed by Prof. Dr. P. Brakman), now part of the IVVO-TNO, Gaubius Laboratory (headed by Prof. Dr. D.L. Knook), Leiden, The Netherlands, under the supervision of Dr. L.M. Havekes. Since November 1989 her research project was funded by The Netherlands Heart Foundation (project # 89.057). In June 1993 the work presented in this thesis was completed.

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge all those who contributed to the realization of this thesis.

I am grateful to Dr. Monique Mulder, with whom I have shared, in the past years, the many ups and downs of an AIO's life. Without the encouragement and support of Peter de Knijff, Elly de Wit and Ninette Tranquille this work would have never been completed. I would also like to mention all my colleagues at the Gaubius Institute who with their cheerfulness and friendship have contributed to a very pleasant atmosphere, especially Allard, Arlène, Bart, Hans, Jaap, Jos and Nicole.

Lenie de Pagter is gratefully acknowledged for providing me with a huge amount of HepG2 cells.

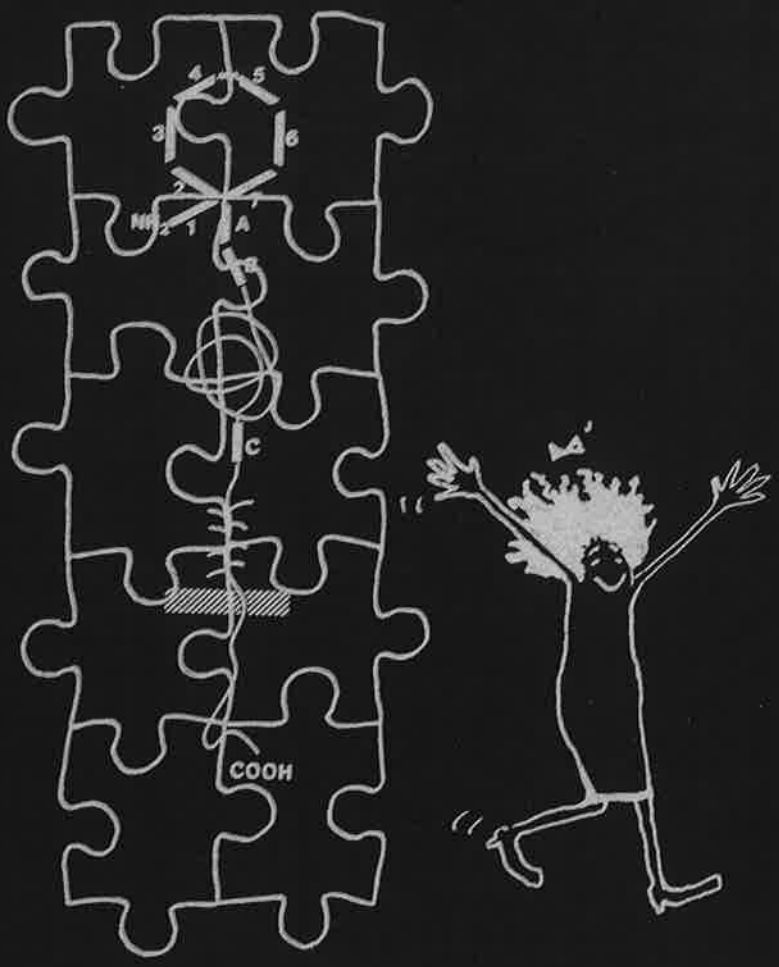
I am grateful to Mariëtte, Arn, André, Inge, Marga, Marten and all the colleagues at the MGC-Department of Human Genetics, Leiden University, for their support.

The support and friendship of Eric Sijbrands, Department of Internal Medicine, University Hospital, Leiden, is much appreciated.

Without the expert secretarial assistance of Marisa Horsting this thesis would never have appeared in this form. Ankie Verwey is acknowledged for her assistance with the literature data base.

I am also grateful to my parents for providing me with the background and insight to obtain this achievement and for their patience and encouragement during the last five years.

Finally, I am particularly indebted to Sergio, my husband, for his emotional and "technical" support. After all, without him, I would have never achieved this Ph.D.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