RETENTION AND ACCUMULATION OF LOW DENSITY LIPOPROTEIN PARTICLES IN THE EXTRACELLULAR MATRIX OF THE ARTERIAL INTIMA IN ATHEROGENESIS

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ACADEMIC DISSERTATION

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Original Publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals.

I. Pentikäinen, M.O., Öörni, K., Lassila, R., and Kovanen, P.T. The proteoglycan decorin links low density lipoproteins with collagen type I. *J. Biol. Chem.* 272:7633-7638, 1997.

II. Pentikäinen, M.O. Öörni, K., Kovanen, P.T. Lipoprotein lipase (LPL) strongly links both native and oxidized low density lipoprotein particles to decorin-coated collagen. Roles for both dimeric and monomeric forms of LPL. *Submitted*, 1999.

III. Pentikäinen, M.O., Lehtonen, E.M.P., and Kovanen, P.T. Aggregation and fusion of modified low density lipoprotein. *J. Lipid Res.* 37:2638-2649, 1996.

IV. Ala-Korpela, M., Pentikäinen, M.O., Korhonen, A., Hevonoja, T., Lounila, J., and Kovanen, P.T. Detection of low density lipoprotein particle fusion by proton nuclear magnetic resonance spectroscopy. *J. Lipid Res.* 1998, 39:1705-1712.

V. Pentikäinen, M.O., Lehtonen, E.M.P., Öörni, K., Lusa, S., Somerharju, P., Jauhiainen, M., and Kovanen, P.T. Human arterial proteoglycans increase the rate of proteolytic fusion of low density lipoprotein particles. *J. Biol. Chem.* 272: 25283-25288, 1997.

In addition, some unpublished data are presented.

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Abbreviations

4-HNE	4-hydroxynonenal
AAPH	2,2'-azobis(2-amidino-propane)hydrochloride
apo	apolipoprotein
BH	Bolton-Hunter
BHT	butylated hydroxytoluene
BM	basement membrane
BODIPY-CE	cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoate
BSA	bovine serum albumin
C-4-S	chondroitin-4-sulfate
C-6-S	chondroitin-6-sulfate
CL	cholesteryl linoleate
CE	cholesteryl ester
CEL	carboxyl ester lipase
CETP	cholesteryl ester transfer protein
CS	chondroitin sulfate
α-CT	α-chymotrypsin
D_2O	deuterium oxide
DAG	diacylglycerol
DS	dermatan sulfate
$DxSO_4$	dextran sulfate
ECM	extracellular matrix
EM	electron microscopy
EC	endothelial cell
FC	free (unesterified) cholesterol
FID	free induction decay
GAG(s)	glycosaminoglycan(s)
^{1}H NMR	proton nuclear magnetic resonance
HDL	high density lipoprotein(s)
HOCl	hypochlorite
HPLC	high pressure liquid chromatography
HS	heparan sulfate
IDL	intermediate density lipoprotein(s)
LDL	low density lipoprotein(s)
IEL	internal elastic lamina
KS	keratan sulfate
LPL	lipoprotein lipase
LRP	low density lipoprotein receptor-related protein
MDA	malondialdehyde
ONOO	peroxynitrite
oxLDL	oxidized LDL
PBS	phosphate buffered saline
PC	phosphatidylcholine
PG(s)	proteoglycan(s)
PL	phospholipid(s)
PLA_2	phospholipase A_2
PLC	phospholipase C
$Pyr_{10}CE$	cholesteryl 1-pyrenedecanoate
RET	resonance energy transfer
SLO	soybean 15-lipoxygenase
SM	sphingomyelin
SMC	smooth muscle cell
SMase	sphingomyelinase
S-SMase	secretory sphingomyelinase
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TG	triglyceride
TSP	sodium 3-trimethylsilyl[2,2,3,3-D_4] propionate
VLDL	very low density lipoprotein(s)
WHHL	Watanabe heritable hyperlipidemia

Summary

Atherosclerosis is characterized by accumulation of low density lipoprotein (LDL)-derived cholesterol in the intima, the inner layer of the arterial wall. Initial extracellular lipid deposition in the subendothelial layer of the arterial intima is followed both by intracellular lipid deposition causing foam cell formation and by the development of an extracellular lipid core deep in the intima. The reason for LDL deposition in the arterial intima is not clear, but appears to be due to formation of modified LDL, which, in contrast to native LDL, tends to accumulate both intra- and extracellularly. LDL modification in the arterial intima, in turn, appears to require binding of the LDL particles to the extracellular matrix, where they are exposed to noxious cell-derived enzymes and agents for prolonged periods of time.

The present study focused on *i*) the interaction of LDL with different components of the extracellular matrix, *ii*) the production of modified forms of LDL morphologically resembling those present in the arterial intima, and *iii*) the effect of arterial proteoglycans on the modification of LDL *in vitro*. Specifically, the binding of LDL to collagen type I in the presence of the small collagen-binding proteoglycan decorin and the enzyme lipoprotein lipase was studied. In addition, LDL particles were modified *in vitro* by mechanical forces, by proteolysis of apolipoprotein B-100 (apoB-100), by lipolysis of LDL surface lipids, by glycosylation of the particles and by oxidation, and the effects of these modifications on the physicochemical properties and integrity of the particles were studied. The proteolytic modification was studied in greater detail in the fluid phase using proton nuclear magnetic resonance spectroscopy and when bound to arterial proteoglycans using fluorescence spectroscopy.

The small proteoglycan decorin was found to bind apoB-100 of LDL by its glycosaminoglycan chain and so link LDL to collagen. Lipoprotein lipase, by binding to both the glycosaminoglycan chain of decorin and to the lipids of LDL, was able to significantly strengthen the binding of LDL to decorin. In addition, even larger amounts of oxidized LDL bound to the decorin-coated collagen in the presence of LPL. This was found to depend on the ability of oxidized LDL to bind to the dimeric form of LPL, whereas native LDL bound only monomeric LPL. Extensive modification of the surface of LDL particles by proteolysis or oxidation, or lipolysis of sphingomyelin was found to generate fused particles resembling the lipid droplets found in the arterial intima. From these modifications, proteolysis also generated membrane material sprouting from the fused particles, which occasionally formed vesicles and multilayered membranous structures. Interestingly, binding of LDL to human arterial proteoglycans was found to increase the rate of proteolytic LDL fusion by enhancing LDL proteolysis. Finally, to monitor fusion of LDL particles and simultaneously to detect changes in the individual classes of surface lipids a novel ^{1}H NMR methodology was devised. Taken together, the present study suggests some plausible mechanisms for the retention and accumulation of LDL in the arterial intima, and has led to the use of novel tools for investigation of the physicochemical properties of native and modified LDL particles.

Introduction

Atherosclerosis is a disease of the inner layer of the arterial wall, the intima. It mainly affects the large arteries and has a strong predilection for certain areas of the arterial tree, *i.e.* the branching sites and bifurcations. Clinical symptoms occur late in the course of the disease and are most commonly caused by obstruction, either partial or total, of the affected artery.

The importance of cholesterol in the pathogenesis of the disease was discovered in the beginning of the century by Anitchkov (1933), who fed rabbits with cholesterol and found that the animals developed atherosclerotic lesions. Today, we know that the major source of the cholesterol in the atherosclerotic lesions is the plasma low density lipoprotein (LDL) particles. Their infiltration into the intima through the endothelial cell layer is followed by their retention in the intima, where they reach very high local concentrations. An important factor contributing to the retention of LDL is its binding to the intimal extracellular matrix. Retention for prolonged periods of time exposes the LDL particles to modifications. Modified forms of LDL in the arterial intima can be detected initially as small lipid droplets and vesicles within the extracellular matrix by electron microscopic techniques, especially in the vicinity of collagen fibers (Fig. 1). They appear to attract monocytes into the intima and lead to formation of foam cells, i.e. intracellular accumulations of lipid. The hallmark of the early atherosclerotic lesion is the presence of lipid-laden foam cells in the intima.

The present study attempts to characterize some of the potential modifications of LDL particles in the arterial intima, and their effect on particle stability and on the interaction of the particles with the extracellular matrix.

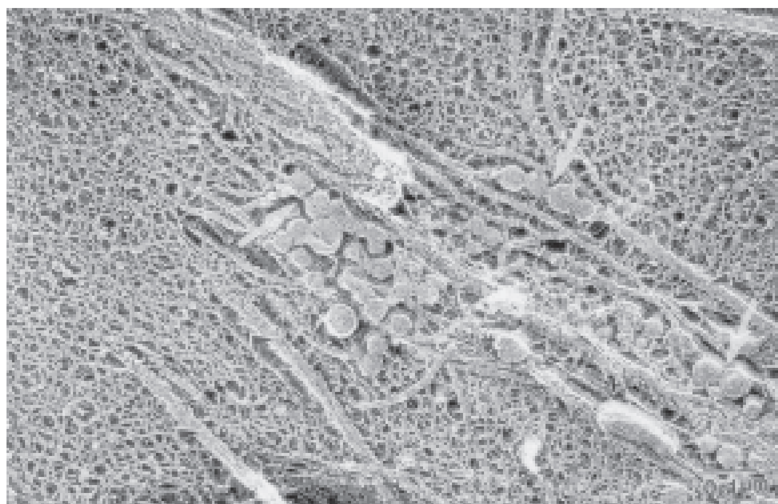

Fig. 1. Freeze-etch electron photomicrograph from the intima of a 3-week-old apoE-deficient mouse aorta. Lipid particles (arrows) are aligned along the collagen fibers (arrowheads). Reproduced from Tamminen *et al.* (1999) with permission of Dr. Joy Frank and the American Heart Association.

Review of the literature

The arterial intima

The normal arterial intima

The arterial wall is composed of three histologically distinct layers. It is the inner layer of the arterial wall, the intima, that is primarily affected by atherosclerosis. This layer is composed of the endothelium with its basement membrane, and of smooth muscle cells, which are the main source of the extracellular matrix of the arterial intima. The intima is separated from the media by the internal elastic lamina (IEL), which is considered to be part of the media. Unlike all other extravascular tissues, the intima lacks lymphatic capillaries (Groszek & Grundy 1980), and this severely limits the removal of macromolecules from the tissue. The middle layer of the arterial wall, the media, is composed of layers of contractile smooth muscle cells separated by elastic laminae. The outermost elastic lamina, called the external elastic lamina, separates the media from the outermost layer of the arterial wall, the adventitia. The adventitia is composed of highly vascularized, loose connective tissue rich in collagen.

The endothelium

The arterial endothelium is composed of a single continuous layer of endothelial cells, which are normally connected with each other by both tight and gap junctions. The endothelial cells are polarized: they secrete a heparan sulfate matrix capable of binding lipoprotein lipase on their apical surfaces, and a basement membrane on their basolateral surfaces.

The endothelium has an important role in regulating the vascular tone. Endothelial cells secrete prostacyclin and nitric oxide, both of which cause relaxation of vascular smooth muscle cells, and endothelin, which causes vasoconstriction. Furthermore, in response to various stimuli from the intima, the endothelial cells express adhesion molecules whose role is to recruit inflammatory cells from the circulation. Finally, the heparan sulfate proteoglycans (HSPGs) on the luminal surface of the endothelial cells bind lipoprotein lipase (LPL), the major lipolytic enzyme involved in the hydrolysis of triglyceride-rich lipoproteins. LPL is secreted by parenchymal cells and not by the endothelial cells themselves. According to a recent report, on their basolateral surfaces endothelial cells express heparinase, which can release LPL in its active form from the underlying extracellular matrix (Pillarisetti *et al.* 1997). Furthermore, endothelial cells can actively transport LPL from their basolateral to their luminal surface (Stins *et al.* 1992).

The endothelium is classically considered to be a major barrier that regulates the entrance of macromolecules into the arterial wall. Despite extensive studies, it is not clear whether macromolecules, such as LDL particles, enter the arterial intima between the normal endothelial cells (Kao *et al.* 1994; Kao *et al.* 1995), or through rare cellular leakage sites associated with endothelial cell turnover (Chuang *et al.* 1990; Barakat *et al.* 1992), or through endothelial cells via transcytosis (Vasile *et al.* 1983; Navab *et al.* 1986; Snelting-Havinga *et al.* 1989). Even the barrier function of the endot-

helium has been questioned, since the LDL concentration in the intima equals or even exceeds the plasma LDL concentration (Smith 1990). In addition, in a model of arterial injury, LDL accumulation in the intima was shown not to be increased in de-endothelialized areas but to be greatly increased in re-endothelialized areas (Day *et al.* 1985; Chang *et al.* 1992).

Intimal cells and stroma

The normal arterial intima consists mainly of smooth muscle cells with a few isolated macrophages. In contrast to the media, where the smooth muscle cells are rich in myofilaments, i.e. are of contractile phenotype, in the intima some of the smooth muscle cells are poor in myofilaments but rich in rough endoplasmic reticulum, i.e., are of synthetic phenotype. These cells are specialized in producing the extracellular matrix of the intima, which consists mainly of proteoglycans, collagens and elastin. In atherosclerotic lesions, the smooth muscle cells especially produce a characteristically thick basement membrane, which is a special type of extracellular matrix (the structure described in “Structure of the extracellular matrix”, p. 16). (Stary *et al.* 1992)

Internal elastic lamina

The innermost continuous elastic lamina of the arterial wall separates the intima from the media and is called the internal elastic lamina (IEL). Similar, although usually thinner, elastic laminae are found between the smooth muscle cell layers of the media. Except for the presence of fenestrae (reported to be as large as ~2 µm in diameter (Kwon *et al.* 1998)), which permit the passage of solvent and small molecules, the IEL is an impermeable barrier. Interestingly, the high density lipoprotein (HDL) particles appear to be able to pass the IEL, whereas LDL particles are not (Nordestgaard *et al.* 1990). Moreover, the IELs of normal arteries have been shown to be impermeable to antibodies (Bendeck *et al.* 1994) and oligonucleotides (Guzman *et al.* 1994). Therefore, the IEL has been suggested to be a molecular sieve that concentrates LDL in the intima (Gofman & Young 1963; Smith 1990; Penn *et al.* 1997).

Morphologic changes in the arterial intima during atherogenesis

In human coronary arteries the arterial intima is especially thickened, which is considered to represent a physiological adaptation to changes in blood flow, wall tension, or both, rather than to be an initial atherosclerotic change. The intima of the coronary arteries is thickened diffusely, except at the branches and orifices, where the thickenings are eccentric. Interestingly, as discussed in more detail below, advanced atherosclerotic lesions tend to develop preferentially at arterial sites with eccentric intimal thickenings. (Stary *et al.* 1992)

A thickened arterial intima characteristically has two distinct layers, which can be detected microscopically. The superficial ”proteoglycan-rich layer” contains large amounts of extracellular matrix consisting of proteoglycans and collagen, isolated smooth muscle cells of synthetic phenotype, and macrophages. The deep ”musculoelastic layer”, in contrast, contains smooth muscle cells of contractile phenotype and elastin arranged as elastic fibers. Although no lipid deposits are evident in the grossly normal intima on light microscopic examination, accumulation of small lipid droplets and vesicles in the extracellular space has been demonstrated already at this stage by electron microscopy (EM) (Tirziu *et al.* 1995).

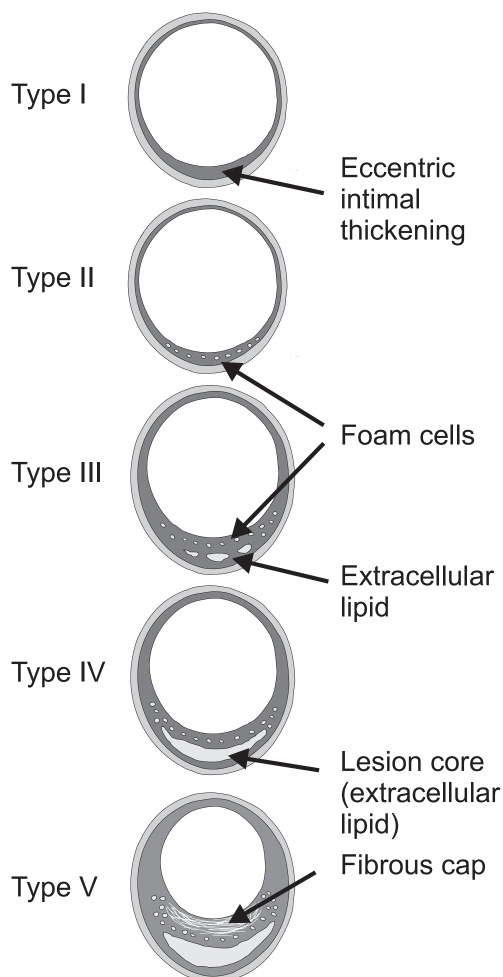

Fig. 2. Changes in the arterial intima during atherogenesis. Adapted from Stary *et al.* (1995).

According to the classification of the American Heart Association (Stary *et al.* 1992; Stary *et al.* 1994; Stary *et al.* 1995), the presence of isolated macrophages that have been transformed into foam cells in the intima is considered to indicate the type I lesion (Fig. 2). Accumulation of large numbers of macrophages with or without lipid deposits as layers in the intima, together with the presence of some lipid-filled smooth muscle cells represent type II lesion. When present in a thin intima, this type of lesion can be seen as a "fatty streak" with the naked eye, but may be undetectable if present deep in a thickened intima. Type II lesions that colocalize with adaptive intimal thickening in atherosclerosis-prone regions may undergo transformation into type III (intermediate) lesions. In these lesions, small pools of extracellular lipid are present in the musculoelastic layer, covered by layers of macrophage-derived foam cells, and some macrophages without lipid deposits are present in the subendothelial space. Confluence of the lipid pools of the type III lesions to form a large lipid core is characteristic of type IV lesions. These lesions have no signs of fibrosis, and the intima above the lipid core appears similar to the intima in type II and type III lesions. However, capillaries that point to the lumen of the vessel are found around the lipid core. The smooth muscle cells in the core have an especially thick basement membrane that separates the cells from the lipid. Formation of fibrous connective tissue around the core is characteristic of type V lesions. Type V_a lesions have a lipid core surrounded by a fibrous cap and are called fibrolipid lesions. Calcified type V_a lesions are termed V_b lesions, and lipid-poor fibrotic lesions, found especially in the lower extremities, are termed V_c lesions. Finally, when type IV or V lesions are complicated by surface disruption, hemorrhage, or thrombosis, the lesions are termed VI_a, VI_b, and VI_c, respectively.

The low density lipoprotein particle

Physiological function of LDL

The bulk of the cholesterol in the plasma circulates in LDL particles. These LDL particles pro-

vide the cells with the cholesterol they need (Brown & Goldstein 1986). They bind to the LDL receptors on the cell surface, after which they are endocytosed (Brown *et al.* 1981). Within lysosomes, acidic proteases hydrolyze the apolipoprotein B-100 (apoB-100) and acidic lipases hydrolyze the lipids of the particles. The cholesterol released from the particles crosses the lysosomal membrane, and is incorporated into the cellular membranes where cholesterol is needed. Uptake of cholesterol by cells is regulated by synthesis of LDL receptors, which prevents intracellular accumulation of cholesterol (Brown & Goldstein 1986).

Structure

LDL particles are spherical lipoproteins with diameters between 18 and 25 nm and densities of 1.019 – 1.063 g/ml. Each particle contains a single apolipoprotein, apolipoprotein B-100 (apoB-100), which partially covers the lipid. LDL particles are heterogeneous, consisting of several subfractions that differ in size, composition, and conformation of apoB-100 (Musliner & Krauss 1988; Austin *et al.* 1988).

Lipids

On average, an LDL particle contains 500 molecules of phosphatidylcholine (PC), 200 molecules of sphingomyelin (SM), 600 molecules of unesterified cholesterol, 1500 molecules of cholesteryl esters (CE), and 200 molecules of triacylglycerols (TG). In addition, a particle contains lipophilic antioxidants, 80% of which is α-tocopherol (on average 6 molecules/particle) (Esterbauer *et al.* 1992).

The phospholipids (PC and SM) and two-thirds of the unesterified cholesterol form an oriented amphipathic surface monolayer on the LDL particles, their polar heads being oriented outward and their nonpolar tails inward (Fig. 3). ApoB-100 has been specifically shown to associate with PC (Sommer *et al.* 1992; Murphy *et al.* 1997), and unesterified cholesterol preferably interacts with SM (Porn *et al.* 1993; Mattjus & Slotte 1996). These findings reveal that lipids are not homogeneously distributed over the surface of LDL particles.

The core of the particles contains the cholesteryl esters, the triacylglycerols, and one-third of the unesterified cholesterol. As illustrated in Fig. 3, 2-6% of the TG and CE penetrate the surface layer (Lund-Katz & Phillips 1986; Kroon 1994). At around 30°C, there is a thermal transition of the core lipids of the LDL particles from a liquid crystalline to a liquid-like state, a process controlled by the amount of TG present in the particles and the degree of saturation of the fatty acids of the cholesteryl ester molecules (Deckelbaum *et al.* 1975; Deckelbaum *et al.* 1977; Kroon 1981).

Analysis of plasma and its isolated lipoproteins by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has revealed a lipoprotein size-dependent shift in the resonances of lipid hydrocarbon chains (Hiltunen *et al.* 1991). This phenomenon has allowed quantification of lipids in different classes of lipoproteins and even in their subclasses (Otvos *et al.* 1992) in plasma in a single measurement (Freedman *et al.* 1998). The basis of this kind of behavior has been explained by a physical lipoprotein model, in which lipoproteins consist of an isotropic core and a radially oriented surface (Lounila *et al.* 1994). (For further details of this model see “Methods”)

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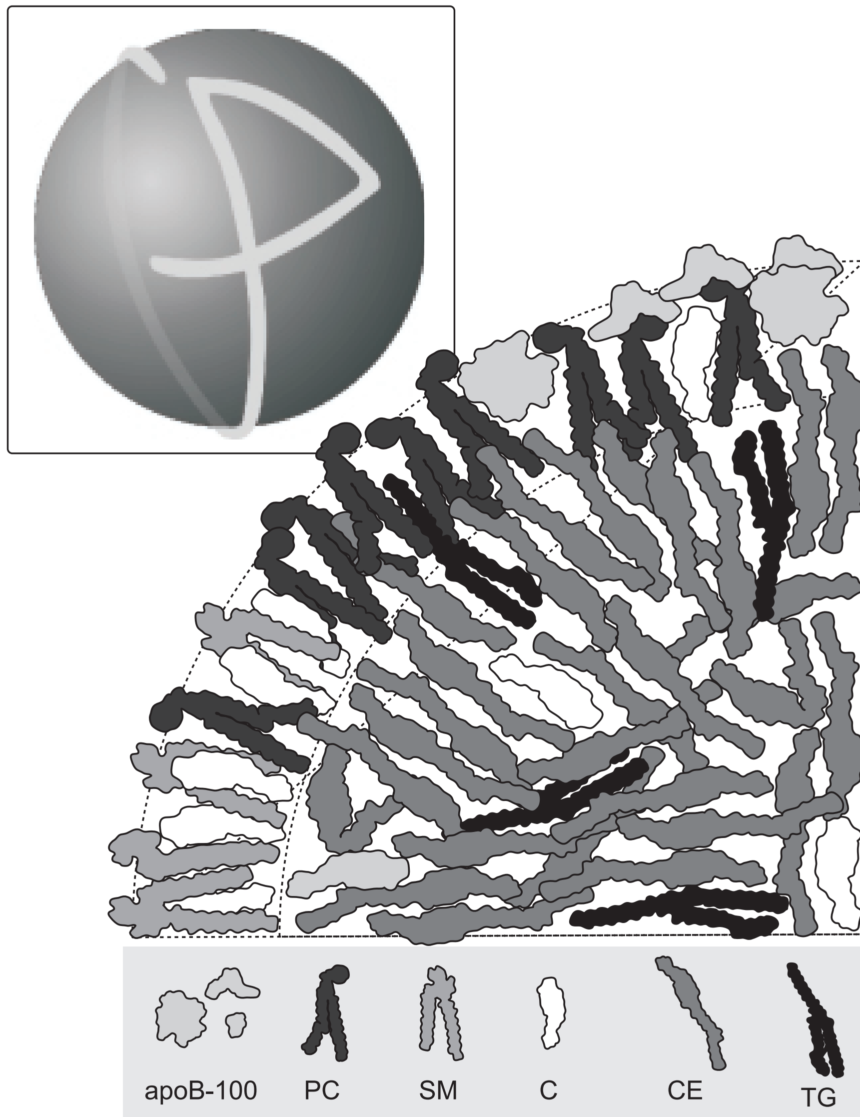

Fig. 3. Schematic representation of the structure of the LDL particle. *Inset* shows folding of apoB-100 on the surface of the LDL particle. Adapted from Borén *et al*. (1998). The main picture shows organization of lipids in a cross-section of the LDL particle. apoB-100, apolipoprotein B-100; PC, phosphatidylcholine; SM, sphingomyelin; C, cholesterol; CE, cholesteryl ester; TG, triglyceride

Apolipoprotein B-100

ApoB-100 consists of 4536 amino acids and has a calculated molecular weight of about 513,000 (Knott *et al.* 1986; Yang *et al.* 1986; Chen *et al.* 1986; Law *et al.* 1986; Cladaras *et al.* 1986). Se-

quence analyses have suggested that apoB-100 has amphipathic α-helices and β-strands organized into a NH_2-α_1-β_1-α_2-β_2-α_3-COOH pentapartite structure. The α_1 domain contains many disulfide bridges, is suggested to be globular in shape, and to be able to associate with lipid. The α_2 and α_3 domains are amphipathic α-helices that can bind lipids reversibly, and the β_1 and β_2 strands bind lipids irreversibly (Segrest *et al.* 1994). Cryo-EM has suggested that apoB-100 forms a ring around the LDL particle (van Antwerpen *et al.* 1997). More detailed analysis by immuno-EM using monoclonal antibodies directed against defined segments on apoB-100 has suggested that 89% of the N-terminal apoB-100 forms a ribbon that wraps around the LDL particle, and the remaining 11% forms a bow that crosses the ribbon (Fig. 3, *inset*) (Chatterton *et al.* 1995). In a recent study by Borén et al. (1998), it was shown with the aid of transgenic animals that it is the arginine residue at 3500 in the "ribbon" that interacts with the C-terminal "bow" of apoB-100 and allows binding of the LDL receptor to residues 3359-3369 of apoB-100.

In addition to binding to the LDL receptor, LDL has been shown to interact with glycosaminoglycans (GAGs) via apoB-100. Analysis of peptides of apoB-100 showed eight potential heparin binding sites (Weisgraber & Rall, Jr. 1987; Hirose *et al.* 1987; Camejo *et al.* 1988). However, in another study by Borén *et al.*, mutation of only a single lysine residues at 3363 was shown to effectively inhibit interaction between apoB-100 and PGs (Borén *et al.* 1998a). Interestingly, lipoproteins comprising only apoB-48, i.e. those lacking the 52% of apoB-100 that contains the proposed PG binding site, also interact with PGs (Borén *et al.* 1998a). Moreover, the N-terminal part of apoB-100 that lacks the proposed PG binding site has been shown to bind to heparin even more strongly than intact apoB-100 (Goldberg *et al.* 1998). Thus, the GAG binding site(s) of apoB-100 are not fully characterized. The N-terminus of apoB-100 has been suggested to bind the enzyme lipoprotein lipase (Sivaram *et al.* 1994; Choi *et al.* 1995; Choi *et al.* 1997), although binding of LPL to the lipids of LDL has also been suggested (Makoveichuk *et al.* 1998). Finally, the C-terminus of apoB-100 has been shown to be important in association with the enzyme platelet-activating factor acyl-hydrolase, a phospholipase A_2 (PLA_2) that hydrolyzes biologically active oxidized phospholipids (PL) of LDL (Tew *et al.* 1996), with LDL particles (Stafforini *et al.* 1999).

Retention of LDL in the intima

Evidence for LDL retention *in vivo*

Increased concentrations of intact undegraded LDL have been detected in early atherosclerotic lesions (Hollander *et al.* 1979; Hoff & Bond 1982; Smith 1990). But is the increased concentration of LDL due to increased influx into the intima or to increased retention in the intima? This question was experimentally addressed by Schwenke and Carew (1989a), who found that the residence time of LDL in a grossly normal arterial intima at sites prone to develop atherosclerotic lesions was dramatically increased. More recently, using a more sophisticated kinetic approach, Tozer and Carew (1997) found no difference in LDL residence time between lesion-resistant and lesion-prone arterial sites without signs of atherosclerosis, but observed a dramatic increase in LDL residence time after the development of fatty streak lesions. The cause of retention of LDL in the ar-

terial intima is not known, but it is widely believed to be due to interaction of LDL with the various components of the extracellular matrix of the arterial intima.

Interaction between LDL and the arterial extracellular matrix

Structure of the extracellular matrix

The extracellular matrix (ECM) gives the vascular wall tensile strength, elastic recoil, compressibility, and viscoelasticity. The intimal cells interact with the extracellular matrix, which regulates cell adhesion, migration, and proliferation. In addition, the extracellular matrix molecules bind plasma proteins, growth factors, cytokines, and enzymes (reviewed by Wight 1996).

As pointed out above, the extracellular space of the arterial intima is composed of large amounts of interstitial ECM, in which collagen and elastic fibers are embedded in a viscoelastic gel containing proteoglycans, hyaluronan, glycoproteins, and water. In addition, a specialized type of ECM, the basement membrane, is present on the basolateral surface of endothelial cells and around smooth muscle cells.

The bulk of the ECM is produced by the smooth muscle cells, although endothelial cells and various inflammatory cells present in the arterial intima have been shown to express various extracellular matrix molecules. Production of ECM by smooth muscle cells can be regulated by other cells of the intima (Edwards *et al.* 1990). Expression of ECM molecules is also regulated by the phenotype of the smooth muscle cells (contractile vs. synthetic), and whether the cells are quiescent or stimulated to proliferate (Nikkari *et al.* 1994; Camejo *et al.* 1993). Interestingly, expression of individual types of ECM molecules are differentially regulated by cytokines (Schönherr *et al.* 1991; Schönherr *et al.* 1993; Edwards *et al.* 1994).

Proteoglycans are composed of glycosaminoglycans (GAGs) that are linked to a core protein. Proteoglycans have been traditionally classified according to their GAGs into chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS) proteoglycans. Staining of the arterial intima for glycosaminoglycans has revealed that the most of the CS is present in the interstitium, whereas DS is associated with collagen fibers, and HS with cell surfaces and with elastin (Völker *et al.* 1986; Völker *et al.* 1987). In atherosclerosis, the amount of HS is decreased and the amount of DS is increased (Wagner & Salisbury 1978; Hollmann *et al.* 1989; Cherchi *et al.* 1990).

Cloning of the core proteins of proteoglycans has allowed characterization of a number of distinct molecules that are characteristically enriched with one type of GAG. Quantitatively, the major PG of the extracellular matrix is the large interstitial CS-rich proteoglycan versican (Yao *et al.* 1994), which comprises ~60-70% of the proteoglycans. In the interstitium, versican interacts with link protein (LeBaron *et al.* 1992) and hyaluronan (Binette *et al.* 1994), and complexes of these molecules fill the interstitial space of the vascular ECM not occupied by cells or the fibrous component (Fig. 4) (Galis *et al.* 1992). The amount and size of the CSPG (versican)-hyaluronan aggregate has been found to be decreased in atherosclerosis (Wagner *et al.* 1983). The ECM also contains the small DS-rich proteoglycans decorin and biglycan. Decorin is found in large amounts in primary atherosclerotic plaques associated with types I and III collagens (Riessen *et al.* 1994). *In vitro*,

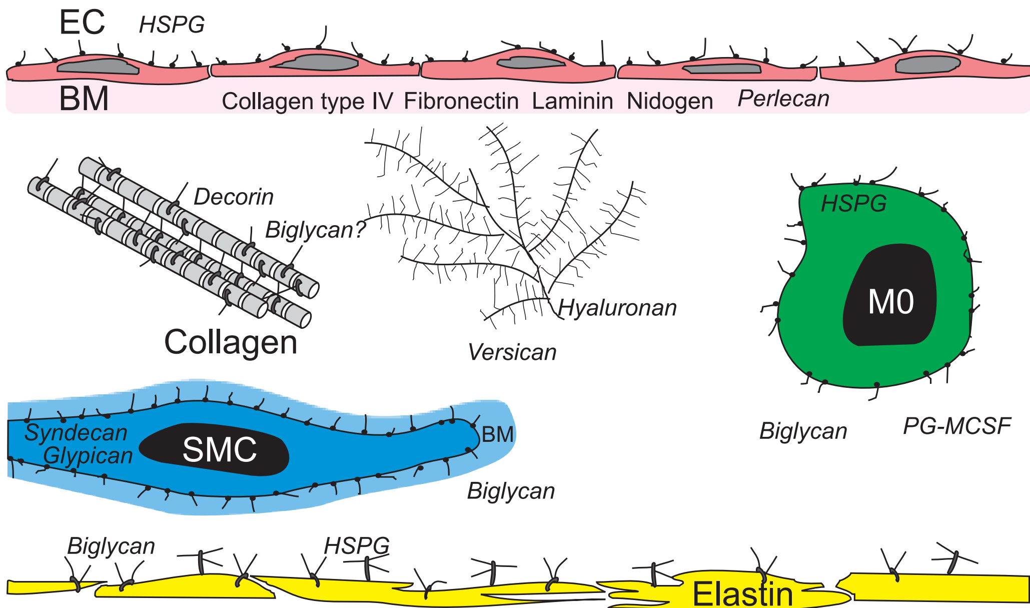

Fig. 4. Organization of extracellular matrix in the arterial intima.
EC, endothelial cell; BM basement membrane; HSPG, heparan sulfate proteoglycan; MO, macrophage; SMC, smooth muscle cell.

decorin has been shown to control collagen fibrillogenesis (Vogel *et al.* 1984). Biglycan, in turn, has been located pericellularly (Riessen *et al.* 1994) and associated with elastin (Evanko *et al.* 1998) in primary atherosclerotic plaques. In contrast to decorin, it has been shown to be greatly increased in amount in restenotic lesions (Riessen *et al.* 1994). *In vitro*, reports on binding of biglycan to collagen are conflicting in that biglycan has been shown either to bind or not to bind to collagen type I (Schönherr *et al.* 1995). Interestingly, the amount of biglycan synthesized by smooth muscle cells in culture was shown to be greatly increased when the cells were treated with oxidatively modified LDL (Chang *et al.* 1996). Perlecan (Murdoch *et al.* 1994), an HSPG containing LDL-receptor domain (binding repeats), is found in basement membranes, which are composed of polymer networks of collagen type IV and laminin, which are linked with entactin/nidogen and by perlecan (Yurchenco & Schittny 1990). In addition, the presence of the KS-rich lumican (Funderburgh *et al.* 1991) and keratocan (Corpuz *et al.* 1996), PG-100 (PG-M-CSF) (Suzu *et al.* 1992), bamacan (Wu & Couchman 1997), and agrin (Tsen *et al.* 1995) in the extracellular matrix have been reported. The surfaces of endothelial cells and smooth muscle cells have been shown to contain the HSPG syndecans 1, 2, and 4 which are attached to the cell via hydrophobic sequences of the core proteins, and glypican-1, which is linked to the cell membranes by phosphatidylinositol linkages (Rosenberg *et al.* 1997).

Interestingly, although the different PGs are generally diffusely located, recent immunostaining

experiments have shown that, in the vascular ring, PGs may be concentrated in quite distinct sectors (Riessen *et al.* 1994; O'Brien *et al.* 1998; Evanko *et al.* 1998; Radhakrishnamurthy *et al.* 1998).

Collagen is the major extracellular protein in advanced lesions, and amounts to up to 60% of the proteins in the lesions. The predominant types of collagen in the intima are types I and III, but the presence of types IV, V, VI, and VIII has also been demonstrated (Mayne 1986). Of note, the basement membranes typically contain large amounts of collagen type IV. Collagen types I and III are organized into distinct fibrillar bundles, which are stabilized by the collagen-binding small proteoglycan decorin, and possibly also by biglycan (Wight 1996).

Elastin in the arterial intima is in the form of elastic fibers, which are complex structures containing elastin protein, microfibrils made of aggregates of proteins and glycoproteins, and GAGs (Pasquali-Ronchetti *et al.* 1995). Staining of GAGs in human arterial intima has shown association of heparan sulfate (HS), chondroitin-4-sulfate (C-4-S), and chondroitin-6-sulfate (C-6-S) with elastin (Wight & Ross 1975; Völker *et al.* 1986; Völker *et al.* 1987; Pasquali-Ronchetti *et al.* 1995). Isolated elastin from the human arterial intima has been shown to contain HSPG and some DSPG (Radhakrishnamurthy *et al.* 1977; Dalferes, Jr. *et al.* 1987; Vijayagopal *et al.* 1983). Although both decorin and biglycan have been shown to be associated with human skin elastin (Baccarani-Contri *et al.* 1990), only biglycan has been shown to be associated with human arterial elastin (Evanko *et al.* 1998). The elastin in atherosclerotic lesions contains large amounts of lipids and calcium, which is likely to derange its elastic properties.

The major **glycoproteins** found in the vascular wall are fibronectin, laminin, thrombospondin, and osteopontin (Wight 1996). They contain multiple domains that allow self-aggregation, and interaction between cells and other components of the extracellular matrix.

Evidence for interaction of LDL with different components of the ECM

Proteoglycans

LDL has been shown to be co-localized with GAGs in the arterial intima in animals and humans (Curran & Crane 1962; Walton & Williamson 1968; Hoff *et al.* 1974; Hoff & Bond 1983; Galis *et al.* 1993). Complexes of lipoproteins and GAGs or PGs have been isolated from human atherosclerotic lesions (Srinivasan *et al.* 1975; Hollander 1976; Woodard *et al.* 1976; Camejo *et al.* 1985a). LDL has been shown to be able to bind to GAGs *in vitro* through binding of positively charged lysine and arginine residues of apoB-100 to the negatively charged sulfate and carboxyl groups of the glycosaminoglycans (Iverius 1972; Camejo 1982). Accordingly, modification of the lysine or arginine residues of LDL by acetylation or by treatment with cyclohexanedione, respectively (Mahley *et al.* 1979), or desulfatation of glycosaminoglycans (Vijayagopal *et al.* 1981) have been shown to block binding of LDL to GAGs. As discussed above in greater detail in "apoB-100" (p. 14) it is surprising that mutation of lysine 3363 to valine in apoB-100 markedly decreased the affinity of LDL for GAGs (Borén *et al.* 1998a). Whether this single lysine is responsible for binding of LDL to GAGs is not known.

The strength of the interaction between apoB-100 and GAGs can be modulated both by GAG-dependent and LDL-dependent factors. When the abilities of different GAGs to bind to LDL were compared, they were arranged in the order heparin > DS > HS > C-4-S (Iverius 1972). The degree of sulfatation appears to be critical, as LDL was shown to bind to C-6-S, which contains oversulfated regions but failed to bind to C-6-S, which lacks oversulfated regions (Sambandam *et al.* 1991). Furthermore, the length of the glycosaminoglycan chains may be of importance, since smooth muscle cells stimulated to proliferate (Camejo *et al.* 1993) and cholesterol-enriched smooth muscle cells (Vijayagopal *et al.* 1996) were shown to produce GAGs of increased size with increased capacity to bind LDL. The large versican-like proteoglycans isolated from atherosclerotic plaques appear to have longer GAG chains than those isolated from normal intima (Wagner *et al.* 1986). Moreover, PGs isolated from atherosclerosis-prone areas of the arterial tree bind increased amounts of LDL (Cardoso & Mourão 1994).

The size of the LDL particles also affects the binding of LDL to GAG, the small dense LDL having the strongest interaction (Hurt-Camejo *et al.* 1990; Anber *et al.* 1997). This has been suggested to depend on a difference in the conformation of apoB-100 between small and large LDL, which favors the binding of small LDL to GAGs. Consistently, LDL isolated from patients suffering from coronary heart disease are enriched in small dense particles and bind more avidly to PGs and GAGs *in vitro* (Camejo *et al.* 1976; Camejo *et al.* 1980; Lindén *et al.* 1989). The sialic acid content of LDL has also been suggested to influence the interaction between LDL and PGs in one study (Camejo *et al.* 1985b) although no association between PG binding and sialic acid content was observed in another study (Anber *et al.* 1997). Recently, aggregation and fusion of LDL particles, two processes that form particles which can bind to GAGs with multivalent apoB-100, have been shown to increase the binding strength to GAGs (Paananen & Kovanen 1994; Paananen *et al.* 1995). In contrast, oxidative modification, which modifies the lysine residues of LDL, has been shown to decrease or block the binding of LDL to GAGs (Öörni *et al.* 1997) and suggested even to release LDL from GAGs (Pentikäinen *et al.* 1997).

Collagen

That LDL particles are associated with collagen fibers has been shown by immunofluorescence (Walton & Williamson 1968; Hoff *et al.* 1974) and by EM techniques (Guyton *et al.* 1985; Pasquinelli *et al.* 1989; Frank & Fogelman 1989; Nievelstein *et al.* 1991; Tamminen *et al.* 1999). Incubation of rabbit aortic leaflets with gold-labeled LDL led to preferential accumulation of the gold particles along collagen fibers (Nievelstein-Post *et al.* 1994). Ultrastructural analysis of collagen fibers by freeze-etch EM revealed that the lipid particles were bound to small fibrils, which were tethered to the collagen fibrils (Fig. 1).

In vitro, native and especially oxidized LDL have been shown to interact with isolated and *in vitro* reconstructed collagen by ionic interactions (Hoover *et al.* 1988; Kalant *et al.* 1991; Kalant & McCormick 1992; Kalant *et al.* 1993; Jimi *et al.* 1994; Greilberger *et al.* 1997). Moreover, glycated collagen has been shown to bind LDL covalently (Brownlee *et al.* 1985). However, the collagen used in these experiments was isolated by methods that had dissociated the collagen-associated proteoglycans present in the arterial intima.

Elastin

The human arterial intima has been shown to contain large amounts of "perifibrous lipid" (Smith *et al.* 1967). Ultrastructural analysis by EM has revealed that this lipid consists of small lipid droplets and vesicles associated with elastin (Guyton *et al.* 1985). Similarly, biochemical measurements have shown an increased lipid content in elastin isolated from atherosclerotic arterial intimas (Claire *et al.* 1976; Sandberg *et al.* 1981; Winlowe *et al.* 1988; Robert *et al.* 1998). Moreover, elastin-lipid complexes have been isolated from atherosclerotic human arterial intima (Srinivasan *et al.* 1980). Staining of neutral lipids and unesterified cholesterol in the intimal elastin has shown that these lipids are deposited to some extent independently, but are mostly colocalized (Bobryshev & Lord 1999).

In agreement with the above-mentioned studies, LDL has been shown to bind to elastin *in vitro* (Kramsch & Hollander 1973; Tokita *et al.* 1977; Srinivasan *et al.* 1980; Noma *et al.* 1982; Noma *et al.* 1983; Winlowe *et al.* 1985; Orekhov *et al.* 1987; Winlowe *et al.* 1988; Podet *et al.* 1991). In these studies, it has been shown that elastin binds LDL lipids rather than apoB-100, and moreover, LDL binds more avidly to elastin extracted from atherosclerotic intimas than to that extracted from normal arterial intimas. Elastin is a strongly hydrophobic substance, and accordingly, lipids are thought to bind to atherosclerotic elastin by hydrophobic interactions.

Molecules that modulate binding of lipoproteins to extracellular matrix and cell surfaces

Lipoprotein lipase (LPL)

LPL is a 55-kD glycoprotein secreted as a catalytically active homodimer. LPL interacts with heparan sulfate and dermatan sulfate GAGs, which stabilizes the dimer, and in the absence of GAGs, it rapidly dissociates into catalytically inactive monomers. LPL has triglyceride lipase activity, which is activated by apoCII and phospholipase A_1 activity.

LPL is the major lipolytic enzyme involved in the intravascular metabolism of postprandial triglyceride-rich lipoproteins. Its physiological function is to hydrolyze the triglycerides of chylomicrons, very low density lipoprotein (VLDL), and intermediate density lipoprotein (IDL) particles on the capillary endothelium, with release of free fatty acids for tissue energy metabolism (reviewed by Goldberg (1996)). There is increasing evidence, however, that LPL is also involved in the pathophysiology of atherosclerosis. Thus, LPL has been shown to be present in the atherosclerotic arterial wall, where it is synthesized by smooth muscle cells and macrophages (Jonasson *et al.* 1987; Ylä-Herttuala *et al.* 1991a; O'Brien *et al.* 1992; O'Brien *et al.* 1994; Semenkovich *et al.* 1998; Araki *et al.* 1998). High levels of LPL secretion in isolated macrophages *in vitro* have been shown to be associated with susceptibility to atherosclerosis in inbred mouse strains (Renier *et al.* 1993). The physiological and pathophysiological function of LPL has been studied in transgenic animals, but the studies have been hindered by the finding that homozygous deficiency of LPL is lethal in the neonatal period (Coleman *et al.* 1995; Weinstock *et al.* 1995). Mice heterozygous for LPL deficiency did not have increased atherosclerosis despite an atherogenic plasma lipoprotein profile, suggesting that decreased levels of LPL in the arterial wall also

protected the mice against atherosclerosis (Semenkovich *et al.* 1998). Finally, the effect of LPL secretion in macrophages in the arterial wall was studied in C57Bl/6 mice on an atherogenic diet, and mice with normal LPL expression in macrophages were found to be more susceptible to atherosclerosis than mice lacking macrophage LPL expression (Babaev *et al.* 1999). This study shows that LPL in the arterial wall is atherogenic, but whether its effect is structural or enzymatic is not yet known.

In vitro, LPL has been shown to bind to HS and DS, but not to CS, collagen, fibronectin, or vitronectin (Saxena *et al.* 1993a). However, differentiated macrophages have been shown to synthesize oversulfated CSPG that can bind LPL (Edwards *et al.* 1995). LPL has been shown to link LDL and VLDL strongly to HS GAGs of basement membranes (Eisenberg *et al.* 1992; Saxena *et al.* 1992; Saxena *et al.* 1993b) and to isolated CS-rich and especially DS-rich PGs from the human aorta (Edwards *et al.* 1993). Oxidation has been shown further to enhance the interaction between lipoproteins and GAGs in the presence of LPL (Auerbach *et al.* 1996; Makoveichuk *et al.* 1998). Although LPL has been reported to bind to the apoB-100 moiety of LDL (Sivaram *et al.* 1994; Choi *et al.* 1995; Choi *et al.* 1997), other studies strongly suggest that LPL actually binds to the surface lipids of LDL, just as it binds to lipid emulsions in the absence of apolipoproteins (Fielding 1969; Lookene *et al.* 1997b; Makoveichuk *et al.* 1998). Finally, pre-injection of LPL has been shown to increase retention of LDL in the walls of perfused microvessels (Rutledge & Goldberg 1994) and aortas (Rutledge *et al.* 1997).

Apolipoprotein E

ApoE is a 34-kD exchangeable apolipoprotein which is present in chylomicrons and their remnants, and in VLDL, IDL, and HDL particles, but not in LDL particles. The N-terminal domain of apoE contains heparin and receptor-binding sites, the C-terminal domain contains amphipathic helices that mediate binding of lipids, and a hinge region connects these two domains. ApoE is a key regulator of plasma lipid levels, affecting both lipoprotein lipolysis and the uptake of lipid particles by the liver. Thus, apoE on the particles at the expense of apoCII inhibits their lipolysis by LPL. ApoE mediates uptake of the particles by cell receptors, which are the LDL receptor, low density lipoprotein receptor-related protein (LRP), and HSPG. (Mahley & Huang 1999)

In addition to regulating lipoprotein metabolism in blood plasma, apoE is likely to be important in the regulation of lipid metabolism in the arterial wall. ApoE is secreted by macrophages in the arterial wall (Basu *et al.* 1981; Chait *et al.* 1982). It is located predominantly on the surfaces of macrophages and in the extracellular matrix surrounding them (O'Brien *et al.* 1994). ApoE, like LPL, binds to HSPG. LPL did not inhibit the binding of apoE to the subendothelial matrix, suggesting that apoE and LPL have different binding sites on the matrix. However, apoE was shown to reduce LPL-mediated retention of LDL in the subendothelial matrix (Saxena *et al.* 1993a). Other reports have shown that LPL and apoE have additive effects on the binding of TG-rich lipoproteins to heparan sulfate (van Barlingen *et al.* 1996; Lookene *et al.* 1997a) and to cells (Mann *et al.* 1999). Interestingly, LPL reduced the secretion of apoE by macrophages (Lucas *et al.* 1997), possibly by sequestering apoE on the cell surface. In addi-

tion to its potential atherogenic effects, apoE may be antiatherogenic because of its ability to mediate cholesterol efflux from cells (Mazzone & Reardon 1994).

The role of arterial apoE in the development of atherosclerotic lesions has been studied in transgenic animals. Expression of human apoE in vascular endothelial cells and smooth muscle cells inhibited diet-induced atherosclerosis in normal mice (Shimano *et al.* 1995) and macrophage expression of human apoE inhibited atherosclerosis in apoE-deficient mice (Bellosta *et al.* 1995). However, elimination of apoE expression in macrophages was shown either to promote (Linton *et al.* 1995) or to inhibit (Boisvert & Curtiss 1999) diet-induced atherosclerosis in normal mice.

Significance of LDL retention

Although various aspects of retention of LDL in the ECM of the arterial wall have been studied for decades, little evidence is available for its causal role in the development of atherosclerotic lesions. However, a number of indirect lines of evidence for the significance of LDL retention have emerged. Retention increases the residence time of LDL in the arterial intima, which allows LDL to be modified. Unlike native LDL, modified LDL can accumulate extracellularly or intracellularly, and these processes are known to be the key elements of atherosclerotic lesions. Moreover, retention of LDL at certain sites in grossly normal aortas was shown to predict subsequent lesion formation in the cholesterol-fed rabbit (Schwenke & Carew 1989b).

Recently, gene technology has made it possible to test the atherosclerotic potential of one aspect of LDL retention, namely interaction with GAGs. Thus, as noted above, Borén *et al.* (1998a) generated transgenic mice expressing mutated human apoB-100, which interacted weakly if at all with glycosaminoglycans *in vitro*. Exposure of these animals to diet-induced hypercholesterolemia caused significantly delayed atherosclerosis compared with mice expressing human apoB-100 (Borén *et al.* 1998b). This strongly suggests that interaction of LDL with glycosaminoglycans in the arterial intima has a causal role in the development of atherosclerosis.

Accumulation of LDL in the arterial intima

Lipids in the atherosclerotic arterial intima

Morphology of lipid particles in the arterial intima

Atherosclerotic plaques contain both extracellular and intracellular lipid deposits. Originally Smith *et al.* observed, with light microscopic techniques, using lipid-soluble dyes, that extracellular lipid droplets were smaller than intracellular lipid droplets (Smith *et al.* 1967). Later, Hoff and Gaubatz (1977) found aggregated LDL-sized and larger spheres positive for apoB in the necrotic core of atherosclerotic plaques. Electron microscopic analysis using lipid-preserving techniques subsequently showed that the extracellular lipids in the arterial lesions are in the form of small droplets containing neutral lipids (diameters 60-200 nm) and vesicles, whereas the intracellular lipid droplets in foam cells are greater than 400 nm in diameter (Bocan *et al.* 1986).

Even the grossly normal human arterial intima has been shown to contain small lipid droplets

and vesicles in the extracellular space (Tirziu *et al.* 1995). In a systematic study in human carotid arteries, Pasquinelli *et al.* showed that the initial lipid deposits were small lipid droplets located subendothelially in the extracellular matrix. These droplets largely disappeared when monocytes entered the intima, where they bacame macrophages, and formed foam cells, suggesting that the foam cells had ingested the droplets (Pasquinelli *et al.* 1989). The presence of initial deposition of extracellular lipid droplets and vesicles before monocyte infiltration has also been shown in experimental animals. Thus, in Watanabe heritable hyperlipidemic (WHHL) and in cholesterol-fed rabbits (Amanuma *et al.* 1986; Simionescu *et al.* 1986; Mora *et al.* 1987a; Frank & Fogelman 1989; Guyton & Klemp 1992), in monkeys (Masuda & Ross 1990), in hamsters (Filip *et al.* 1987), and in apoE-knockout mice (Tamminen *et al.* 1999), small extracellular lipid droplets and vesicles have been observed in the ECM of the arterial intima very soon after exposure to hypercholesterolemia. Importantly, Mora et al. found that accumulation of apoB and lipids in the arterial intima coincided both spatially and temporally, strongly suggesting that these lipids are derived directly from apoB-containing lipoproteins (Mora *et al.* 1989b). Enlarged lipid particles have been observed in the extracellular matrix of rabbits 2 hours after infusion of large amounts of human LDL (Nievelstein *et al.* 1991), showing rapid fusion of human LDL particles into the small lipid droplets in the extracellular matrix *in vivo*. A similar finding was made in rabbit aortic leaflets incubated for 4 hours with human LDL *in vitro* (Nievelstein-Post *et al.* 1994).

Whether the lipid core of an advanced atherosclerotic lesion develops from degeneration of foam cells in a fatty streak lesion or by direct deposition of lipid is still open to debate. The foam cells in fatty streak lesions are located in the superficial intima, whereas the lipid core of a fibrolipid lesion develops deep in the musculoelastic layer of the intima. Guyton *et al.* have shown that lipids in the early core regions of transitional small fibrolipid lesions were mostly composed of small vesicles within the extracellular matrix deep in the musculoelastic layer beneath the foam cells. Upon development of a fibrous plaque, the lipid deposition varied: in some plaques, vesicles and cholesterol crystals were the predominant form of lipid deposit in the core, but, in other fibrous plaques, small lipid droplets predominated, with a notable absence of cholesterol crystals. Interestingly, it appeared that foam cells were more consistently present in caps of fibrolipid lesions rich in vesicles and cholesterol crystals than in caps of fibrous plaques rich in small lipid droplets. (Guyton & Klemp 1994) Thus, it appears that accumulation of cholesterol in the cores of early lesions is dependent on the presence of macrophage foam cells.

Chemistry of lipids in the arterial intima

The predominant lipids accumulating in the arterial intima are cholesteryl esters, unesterified cholesterol, and phospholipids. Interestingly, their accumulation appears to be both temporally (Guyton & Klemp 1992; Chao *et al.* 1994; Guyton & Klemp 1994) and spatially (Kruth 1984a; Kruth 1984b) distinct. Although plasma triglyceride-rich VLDL and IDL particles, and possibly also chylomicron remnants, have been suggested to participate in the lipid accumulation, triglycerides have been shown to account for only ~1% of the lesion lipids (Rokosova *et al.* 1986).

Comparison of the composition of lipids of the arterial wall with plasma LDL particles has been made and the results are compatible with the notion that the major source of the lipid in the atherosclerotic lesions is plasma low density lipoprotein particles. Smith *et al.* made the interesting original observation that the fatty acid composition of cholesteryl esters in areas possessing either intra- or extracellular lipids deposits differed markedly, the former being enriched in oleate and the latter in linoleate like the plasma LDL (Smith *et al.* 1968). That intracellular lipids rich in cholesteryl oleate can also be derived from plasma LDL rich in cholesteryl linoleate has later been shown to be possible *in vitro*. Thus, LDL taken up by cells via the LDL-receptor is targeted into lysosomes, where its cholesteryl esters are hydrolyzed. The liberated unesterified cholesterol is transported into the cytosol, where it is re-esterified by acyl-coenzyme A:cholesterol acyltransferase, which has a preference for oleyl coenzyme A over linoleyl coenzyme A (Brown & Goldstein 1983). Taken together, the intracellular lipids may be envisioned as being derived either directly from plasma LDL or from extracellular lipid particles. The extracellular lipid particles, however, are not likely to be derived directly from the intracellular cytoplasmic deposits.

The proportion of cholesterol in esterified form in a lesion was recently correlated with the ultrastructure of the lesion core. The lesions rich in small vesicles and cholesteryl crystals were highly enriched in unesterified cholesterol, whereas, in the cores of lesions with predominance of small lipids droplets, up to 95% of the cholesterol was esterified (Guyton & Klemp 1994).

The presence of oxidized lipids, notably oxysterols, in the arterial wall has been recognized a long time ago (Brooks *et al.* 1966). Although oxysterols seem to be present only in small amounts, their presence may be significant because of their potent effects on cells (Chisolm *et al.* 1994; Hulten *et al.* 1996). Recent careful analysis of the oxidation state of lipids and antioxidants has shown that the normal intima almost totally lacks oxidized lipids, whereas in atherosclerotic plaques, ~30% of cholesteryl linoleate is in oxidized form despite the presence of large amounts of water-soluble and lipid-soluble antioxidants (Suarna *et al.* 1995).

Evidence for the presence of modified LDL *in vivo*

Although alterations in LDL can occur in the blood plasma (Avogaro *et al.* 1988), possibly induced by a recently described transsialidase (Tertov *et al.* 1998; Tertov *et al.* 1995; Demuth *et al.* 1996), it is widely accepted that the major modifications of LDL occur in the arterial intima, where the LDL particles may have lost the protection against modification afforded by the plasma antioxidants and protease inhibitors.

Immunological evidence

Torzewski *et al.* produced monoclonal antibodies against LDL modified by trypsin, cholesterol esterase, and neuraminidase. One of the antibodies reacted *in vitro* with trypsin-modified LDL, whereas the other reacted with LDL modified by a combination of trypsin and cholesteryl esterase. Both of these antibodies reacted with epitopes in human arterial intima that were colocalized with the terminal complement (Torzewski *et al.* 1998). The presence of oxidatively modified (lipo)proteins both in the circulation and in the arterial intima has been stud-

ied extensively. Autoantibodies against LDL modified by malondialdehyde (MDA), copper, peroxidase, and 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH) have been found in the circulation (Palinski *et al.* 1989; Salonen *et al.* 1992; Seccia *et al.* 1997). In the arterial intima, epitopes can be detected with antibodies against MDA-modified LDL (Haberland *et al.* 1988; Palinski *et al.* 1989), 4-hydroxynonenal (HNE)-modified LDL (Palinski *et al.* 1989), oxidized phospholipids (Itabe *et al.* 1994), hypochlorite-modified LDL (Hazell *et al.* 1996), and nitrotyrosine (Beckmann *et al.* 1994). Furthermore, immunocomplexes of oxLDL have been detected in the arterial intima (Ylä-Herttuala *et al.* 1994). Finally, different advanced glycosylation end products (AGEs) have been detected immunohistochemically, and found to colocalize with oxidized LDL (Sakata *et al.* 1998).

Evidence based on properties of lipid particles isolated from the arterial intima

Methodological aspects of extraction

In most studies, lipoproteins have been isolated from cadavers, which may have resulted in post-mortem artifacts. In fact, it was recently shown that apoB-100 of LDL isolated from a fresh surgical sample was minimally fragmented, whereas the LDL isolated from an aorta obtained at autopsy was extensively fragmented (Rapp *et al.* 1994). Significant differences may also result from the extraction method used. Thus, mild buffer extraction of minced tissue produces the fewest artifacts, but has a low yield. The yield can be increased by disruption of the tissue by homogenization (Hoff *et al.* 1991) or with hydrolytic enzymes (Hoff & Gaubatz 1979), but these treatments give rise to modification of the particles. Homogenization has been shown to convert LDL into larger (Hoff *et al.* 1991) and smaller lipid particles (Kruth 1997). Hydrolytic enzymes (collagenase, chondroitinase, and elastase) degrade apoB-100, precluding analysis of the intactness of apoB-100, and may also convert LDL into larger particles (Piha *et al.* 1995; Kruth 1997). Finally, tightly bound LDL has been released by the combination of homogenization and detergent extraction (Hoff *et al.* 1978b; Hoff *et al.* 1978c), but the material released is suitable only for protein quantitation.

Properties of isolated lipid particles

Lipid particles extracted from the arterial intima can be grossly divided into four categories: (1) small lipid vesicles rich in unesterified cholesterol and lacking immunoreactive apoB-100, (2) small lipid droplets rich in esterified cholesterol and lacking immunoreactive apoB-100, (3) arterial IDL/VLDL, i.e. lipoproteins containing both apoB-100 and apoE that have the density of plasma VLDL and are larger than plasma LDL, but, in contrast to plasma VLDL and IDL, are triglyceride-poor, and (4) lipoproteins closely resembling plasma LDL. In advanced atherosclerotic lesions, the proportion of cholesterol in particles containing immunoreactive apoB-100 has been estimated to be only ~5% (Hoff *et al.* 1978a). Therefore, unless LDL is carefully isolated, the "arterial LDL" preparation will be contaminated with lipid particles lacking apoB-100 that may or may not be derived directly from plasma LDL particles. Therefore, analysis of the properties and composition of LDL in the arterial intima should be restricted to LDL isolated from fresh arterial intimas by a combination of immuno-affinity chromatography and either gel filtration or density gradient ultracentrifugation, since other lipid particles have

been shown to have densities overlapping that of LDL particles (Chao *et al.* 1994).

1. Unesterified cholesterol-rich vesicles

Chao *et al.* and Tirziu *et al.* have isolated and characterized unesterified cholesterol-rich vesicles from the arterial intima (Chao *et al.* 1988; Chao *et al.* 1990; Chao *et al.* 1994; Tirziu *et al.* 1995). The density of the vesicles ranged between 1.01 g/ml, and 1.05 g/ml. They contained no immunoreactive apoB-100, but had albumin in their cores. Cholesterol in the vesicles was mostly unesterified, and the predominant phospholipid was found to be sphingomyelin. Electron microscopy confirmed that the particles were uni- and multilamellar vesicles, and the presence of albumin in the core of the vesicles strongly suggested that they had been formed extracellularly.

2. Cholesteryl ester-rich lipid droplets

Chao *et al.* (1990) isolated and purified cholesteryl ester-rich lipid particles from minced human aortas by microfiltration, gel filtration chromatography, and density gradient ultracentrifugation. These particles correspond to the small lipid droplets containing neutral lipids detected microscopically (Kruth & Shekhonin 1994; Guyton & Klemp 1988). The particles had a density <1.01 g/ml; 76% of their cholesterol was esterified, linoleate was the major fatty acid esterified to cholesterol, and the particles contained only small amounts (<10%) of phospholipids, most of which was sphingomyelin. Electron microscopy confirmed that these particles were small spherical lipid droplets having a surface monolayer with diameters between 40 and 200 nm. Scanning EM revealed the presence of pits on the surface of the particles, which is consistent with other reports on lipid particles of a similar kind isolated from the arterial intima (Hollander *et al.* 1979; Guyton *et al.* 1990).

3. Arterial VLDL/IDL

Rapp *et al.* (1994) and Ylä-Herttuala *et al.* (1988) found apoB-100- and apoE-containing lipoproteins in the VLDL-IDL density range, which, in contrast to plasma VLDL and IDL particles, were poor in triglycerides and rich in cholesteryl esters. The high content of apoE could reflect the presence of infiltrated β-VLDL-like particles, or the acquisition of apoE from tissue macrophages (Basu *et al.* 1982). Immunohistochemical demonstration of apoC-III, a component of plasma VLDL particles, in human atherosclerotic plaques suggests that the particles could have infiltrated from plasma (Hoff *et al.* 1976). However, the notable absence of apoE in atherosclerotic lesions in mice lacking macrophage apoE secretion (Fazio *et al.* 1997; Boisvert & Curtiss 1999) suggests that most of the apoE in the atherosclerotic intima is due to local synthesis, rather than to plasma infiltration. ApoB-100 of the arterial VLDL/IDL was found to be nearly intact (Rapp *et al.* 1994). Electron microscopy showed that the particles were solid spheres of the size of plasma VLDL and larger (Rapp *et al.* 1994).

4. Arterial LDL

LDL isolated from surgical samples of atherosclerotic intima by a combination of immunoaffinity chromatography and density gradient ultracentrifugation closely resembled plasma LDL particles. Thus, in contrast to previous reports, which included particles not containing immunoreactive apoB, the arterial LDL did not differ from plasma LDL in lysoPC, SM, or PL content (Tailleux *et al.* 1993), or FC/TC or TC/TG ratios (Rapp *et al.* 1994). Interestingly, LDL isolated from atherosclerotic lesions was shown to be 10 to 50-fold enriched in ceramide, a cleavage product of sphingomyelin, and ceramide was found to be present only in aggregated forms of

LDL (Schissel *et al.* 1996). In contrast, no diacylglycerol, a product of PC cleavage by phospholipase C, was found. Electron microscopy showed that the arterial LDL particles were solid spherical particles larger than plasma LDL (Rapp *et al.* 1994). In addition, density gradient ultracentrifugation showed that the LDL-sized particles had a slightly lower and more heterogeneous hydrated density than plasma LDL (Morton *et al.* 1986).

ApoB-100 in LDL isolated from cadaveric normal human arterial intima has been shown to be almost intact (Ylä-Herttuala *et al.* 1988; Steinbrecher & Lougheed 1992), but in many reports, apoB-100 of LDL isolated from a cadaveric atherosclerotic arterial wall has been shown to be hydrolyzed to variable degrees (Clevidence *et al.* 1984; Daugherty *et al.* 1988; Ylä-Herttuala *et al.* 1989; Hoff & O'Neil 1991; Steinbrecher & Lougheed 1992; Tailleux *et al.* 1993). However, LDL, IDL, and VLDL isolated from surgical samples of the human atherosclerotic arterial intima by buffer extraction and then an anti-apoB column showed minimal hydrolysis of apoB-100, whereas lipoproteins isolated from cadaveric aortas 10 hours after death, even in the presence of protease inhibitors, showed extensive degradation of apoB-100 (Rapp *et al.* 1994). Thus, it appears that detection of hydrolyzed apoB-100 in LDL isolated from cadaveric atherosclerotic intima even in the presence of protease inhibitors and antioxidants should be viewed with caution. Hydrolysis of apoB-100 is not necessarily a marker of proteolytic cleavage since oxidation has also been shown to cleave apoB-100 (Schuh *et al.* 1978).

Arterial LDL has been shown to have an increased electrophoretic mobility relative to plasma LDL (Hoff & Gaubatz 1982). Shaikh *et al.* showed that the electrophoretic mobility of LDL is increased *in vivo* in the arterial intima as little as 24 hours after injection of radiolabeled LDL into humans. This increase in electrophoretic mobility is not associated with hydrolysis of apoB-100, which suggests that it may not be dependent on lipid peroxidation (Shaikh *et al.* 1991). In fact, Ylä-Herttuala *et al.* showed that LDL obtains an increased electrophoretic mobility even when mixed with minced arterial tissue (Ylä-Herttuala *et al.* 1990b).

Oxidative damage to arterial LDL has been detected by various more or less specific markers. Thus, LDL isolated from aortas of WHHL rabbits was shown to have an increased amount of thiobarbituric acid-reactive substances (Daugherty *et al.* 1988). Arterial LDL cross-reacted with an antibody raised against malondialdehyde modified LDL (Haberland *et al.* 1988). LDL isolated from atherosclerotic lesions had fluorescent properties similar to LDL oxidized *in vitro* (Hoff & O'Neil 1991). More specific markers of oxidative damage that are unique for a distinct type of oxidative damage have also been determined. Thus, *o′*, *o′*-dityrosine, produced from tyrosine by myeloperoxidase (Heinecke *et al.* 1993) was increased 100-fold in LDL isolated from atherosclerotic lesions as compared with plasma LDL (Leeuwenburgh *et al.* 1997b). 3-Chlorotyrosine, another marker of myeloperoxidase-mediated oxidation, was markedly elevated in LDL isolated from atherosclerotic plaques (Hazen & Heinecke 1997). Lipoxygenase oxidation products have been detected in early atherosclerotic lesions of rabbits (Kühn *et al.* 1994) and humans (Kühn *et al.* 1997) and in human atherosclerotic plaques (Folcik *et al.* 1995). Elevated levels of nitrotyrosine, a marker of reactive nitrogen spe-

cies, were found in LDL isolated from the arterial intima (Leeuwenburgh *et al.* 1997a). Finally, *o* and *m*-tyrosine, markers of metal-ion damage, are increased in LDL isolated from advanced atherosclerotic plaques (Leeuwenburgh *et al.* 1997b)

In summary, the arterial intima contains plasma-derived LDL particles that showing signs of modification of several different types. Moreover, the lipid particles, in the form of both vesicles and lipid droplets, have features suggesting that they are derived from plasma LDL by extensive modification.

Evidence for the presence of enzymes and agents potentially capable of modifying LDL *in vivo* in the extracelular space of the arterial intima

Proteases

Mast cells have been shown to be present in the normal and atherosclerotic arterial intima, and to contain the proteolytic enzyme chymase capable of degrading apoB-100 (Kaartinen *et al.* 1994). The arterial intima has been suggested to contain large amounts of plasmin (Hendriks *et al.* 1998), also capable of cleaving apoB-100. Lysosomal proteases of macrophages have been shown to degrade apoB-100 at acidic pH (Leake *et al.* 1990). Finally, atherosclerotic plaques contain a number of specific enzymes capable of degrading the extracellular matrix, e.g. matrix metalloproteinases (Henney *et al.* 1991; Galis *et al.* 1994; Galis *et al.* 1995; Sukhova *et al.* 1998), which are also capable of degrading apoB-100 to some extent (Kruth 1997).

Oxidants

The cells in the normal arterial intima express nitric oxide synthetase and cyclooxygenase I constitutively. The cells in atherosclerotic lesions also express an inducible form of nitric oxide synthetase, NADPH oxidase, and cyclooxygenase II, which produces free radicals potentially capable of oxidizing LDL (Ylä-Herttuala 1998). In addition, the arterial intima has been shown to express the enzymes 15-lipoxygenase (Ylä-Herttuala *et al.* 1990a; Ylä-Herttuala *et al.* 1991b), myeloperoxidase (Daugherty *et al.* 1994), and heme-oxygenase-1 (Wang *et al.* 1998) capable of enzymatic oxidation of LDL. Finally, atherosclerotic plaques have been shown to contain transition metals potentially capable of oxidizing LDL nonenzymatically (Smith *et al.* 1992; Evans *et al.* 1995; Lamb *et al.* 1995). Interestingly, iron was shown to colocalize with ceroid, an insoluble complex of oxidized lipid and protein, in human atherosclerotic lesions (Lee *et al.* 1998).

Lipases

Secretory nonpancreatic PLA_2 is present in the human arterial intima (Menschikowski *et al.* 1995; Hurt-Camejo *et al.* 1997), being especially located along collagen fibers (Romano *et al.* 1998). This type of PLA_2 has been shown to be active against human LDL, and its activity is increased by GAGs (Sartipy *et al.* 1996; Sartipy *et al.* 1998).

The arterial wall has been shown to possess SMase activity, which increases during atherogenesis (Rachmilewitz *et al.* 1967; Portman & Alexander 1970). The arterial enzyme has been identified as secretory SMase (S-SMase) (Schissel *et al.* 1996). Recently, macrophages (Schissel *et al.* 1996) and human vascular endothelial cells (Marathe *et al.* 1998) have been

shown to express this enzyme. S-SMase has an acid pH optimum, but can hydrolyze SM of oxidized LDL, and LDL treated with PLA_2 or enriched with apoCIII *in vitro* (Schissel *et al.* 1998). Interestingly, S-SMase was recently shown to associate with laminin and collagen *in vitro* (Marathe *et al.* 1999)

The arterial intima has been shown to contain bile acid-stimulated cholesterol esterase activity. This activity could be precipitated with antibody raised against the enzyme carboxyl ester lipase (CEL) (Shamir *et al.* 1996). CEL is a pancreatic lipolytic enzyme that exhibits bile acid-independent lysophospholipase activity and bile acid-stimulated cholesterol esterase activity. CEL was recently shown to be able to hydrolyze lysophosphatidylcholine (lysoPC) in oxidized LDL, and cholesteryl esters in LDL in the presence of circulating levels of bile acids (Shamir *et al.* 1996). Interestingly, endothelial cells and macrophages have been shown to secrete CEL in culture (Li & Hui 1997; Li & Hui 1998). Endothelial cells in culture (Sando *et al.* 1990), and macrophages and macrophage-derived foam cells in the vessel wall have been shown to synthesize lysosomal acid lipase (Davis *et al.* 1985). Whether this enzyme is active in the intimal extracellular space is not known.

Finally, endothelial cells have been shown to secrete a novel LPL-like lipolytic enzyme (Jaye *et al.* 1999; Hirata *et al.* 1999) that has phospholipase A_1 activity but no triglyceride lipase activity. Overexpression of this enzyme in mice was capable of decreasing HDL levels (Jaye *et al.* 1999). Whether this lipase is also involved in LDL metabolism remains to be studied.

Aggregation and fusion of modified LDL particles *in vitro*

Methodological aspects

Aggregation and fusion of LDL have been detected by various methods based on the physicochemical properties of the particles and on particle morphology, but little effort has been devoted to distinguishing these two processes.

The turbidity of the lipoprotein solution, which can be caused by either aggregation or fusion, has been used in many studies as an indicator of particle size (Khoo *et al.* 1988). Turbidity is easy and quick to measure, which enables measurement of large aggregates. However, it is nonquantitative and very nonspecific. Gel filtration of lipoprotein particles, using columns that allow separation of native LDL from larger structures, has also been used in many studies (Piha *et al.* 1995). The advantages of gel filtration are that it is quantitative, sensitive to small increases in particle size, and reproducible. However, it lacks the specificity to distinguish aggregation from fusion, and recovery of large lipid particles is often poor. Rate zonal flotation of LDL (Polacek *et al.* 1988) has the advantage of almost complete recovery of LDL from the gradients, but may give false results if the density of the particles has been considerably changed during LDL modification. Dynamic laser scattering has also been used to measure lipoprotein size, but appearsto be unable to distinguish fused from aggregated particles (Kawabe *et al.* 1994).

Electron microscopic techniques are able to distinguish particle fusion from aggregation and to reveal gross changes in particle morphology. However, electron microscopy is not quantitative, is time-consuming, and has many poten-

tial artifacts. Negative staining (Forte & Nordhausen 1986) of lipoprotein preparations is rapid and allows measurement of particle size. However, large aggregates of LDL are not visible, staining of the grid is usually highly variable, and usually only a small proportion of the grid can be examined. Therefore, there is a danger of analyzing a selected population of the sample. Casting of the lipoprotein sample into agarose and staining of thin sections with lipid preserving techniques (Guyton *et al.* 1991; Steinbrecher & Lougheed 1992) gives superior morphological images of modified LDL particles and allows analysis of LDL particles even in large aggregates. However, native-sized LDL particles are poorly visible and their retention in the gel during processing is not guaranteed.

Taken together, the methods used to quantify LDL particle aggregation and fusion in the literature have several drawback. Therefore, extensive efforts have been made in the present study to develop and validate methods for studying LDL particle aggregation and fusion.

Modifications that cause aggregation and fusion of LDL particles *in vitro*

Mechanical stress induced by vortexing of LDL has been shown to cause turbidity of an LDL sample (Khoo *et al.* 1988). Ultrastructural EM analysis has shown that vortexed LDL is in the form of aggregated native-sized LDL particles, small lipid droplets and vesicles (Guyton *et al.* 1991). The effect of vortexing could be inhibited by exchangeable apolipoproteins (apoE, apoC, and apoA-I) that, in contrast to phospholipase C-induced aggregation, were not associated with LDL after vortexing (Khoo *et al.* 1990). Thus, it appears that, during exposure of LDL to mechanical stress, hydrophobic spots emerged. In the absence of exchangeable apolipoproteins, these spots can trigger particle aggregation and fusion, and binding of exchangeable apolipoproteins to these spots can inhibit aggregation and fusion of LDL. The *in vivo* significance of this fusion induced by mechanical stress is possible but, at present, is not shown.

Proteolytic degradation of apoB-100 of LDL has been shown to cause aggregation and fusion of LDL. Thus, degradation of LDL with neutrophil elastase has been shown to cause dimerization of LDL particles (Polacek *et al.* 1988). LDL particle fusion was first observed on the surface of mast cell granules to which both the neutral proteases chymase and carboxypeptidase A and also LDL were bound (Kokkonen & Kovanen 1989). Experiments with a number of different proteases have later shown that release of fragments of apoB-100 is a prerequisite for LDL fusion (Piha *et al.* 1995). ApoB-100 covers ~30% of the surface volume of LDL, and proteolysis of apoB-100 must lead to reorganization of both surface and core lipids of LDL, resulting in fusion of the particles. This is supported by the finding that, despite extensive proteolysis, fusion of proteolyzed LDL is slow at 15°C, a temperature at which the core lipids of the LDL particles are highly organized and reorganization of LDL lipids is hindered (Paananen & Kovanen 1994).

Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyzes phospholipids into lysophospholipids and free fatty acids. In the presence of albumin, most of the free fatty acid and some of the lysophospholipids leave the LDL particles (Kleinman *et al.* 1988). Treatment of LDL with *bee venom* PLA_2 in the presence of albumin has been shown to lead to formation of

aggregated LDL particles which, in electron microscopy, appear to be smaller than native LDL particles (Öörni *et al.* 1998). The decrease in particle size is thought to be due to reorganization of LDL lipids (Kleinman *et al.* 1988; Gorshkova *et al.* 1996), which leads to increased interpenetration of surface and core lipids (Gorshkova *et al.* 1996). In contrast, PLA_2 treatment of LDL in the presence of heparin was shown to result in fusion of LDL particles (Hakala *et al.* 1999). The fact that fusion was observed even if LDL was pretreated with heparin and lipolyzed in the absence of heparin, suggests that heparin caused an irreversible conformational change in apoB-100 which promoted LDL particle fusion after lipolysis.

Phospholipase C (PLC) hydrolyzes phospholipids into phosphocholine and diacylglycerol (DAG). Phosphocholine is hydrophilic and is released from the LDL particles, whereas DAG is hydrophobic and appears to be able to reside both in the surface and in the core of the particles (Wang *et al.* 1995). PLC treatment of LDL has been shown to result in instability of the particles (Pollard *et al.* 1969), increased turbidity of the sample (Suits *et al.* 1989), and both aggregation and fusion of the particles (Liu *et al.* 1993). The aggregation and fusion of the PLC-treated LDL particles appears to be caused by formation of a hydrophobic surface on the LDL particles, a phenomenon that depends on both an increase in the neutral lipid content of the particles by DAG and loss of surface phospholipids (Singh *et al.* 1992). Consistently, exchangeable apolipoproteins A-I, E (Khoo *et al.* 1990), and *Manduca sexta* apolipophorin III (Liu *et al.* 1993) have been shown to inhibit PLC-induced aggregation and fusion of LDL by binding to the particles and potentially covering the hydrophobic domains formed. The physiological significance of PLC treatment is uncertain, since LDL isolated from an atherosclerotic intima does not contain detectable amounts of DAG (Schissel *et al.* 1996) and no evidence has been found for the presence of secreted PLC in the arterial intima.

Sphingomyelinase (SMase) hydrolyzes the sphingomyelin (SM) molecules in LDL particles into phosphocholine molecules, which are released from the particles, and ceramide molecules, which are retained in the particles (Schissel *et al.* 1996). Treatment of LDL with SMase from *Bacillus ceraus* has been shown to induce both aggregation and fusion of LDL particles (Xu & Tabas 1991; Paananen & Kovanen 1994). This has been shown to depend on the increase in the ceramide content and to be independent of the SM content of particles (Schissel *et al.* 1996). The mechanism could involve microdomain formation of ceramide molecules (Holopainen *et al.* 1998) that can act as hydrophobic spots. This is supported by the finding that treatment of LDL with sphingomyelinase at 15°C, which allows sphingomyelin hydrolysis, but hinders lateral diffusion of surface phospholipids, did not lead to particle aggregation or fusion (Öörni *et al.* unpublished).

Oxidation by copper (Hoff & O'Neil 1991), AAPH (Kawabe *et al.* 1994), and hypochlorite (HOCl) (Hazell & Stocker 1993; Hazell *et al.* 1994) have been shown to cause aggregation of LDL. Interestingly, modification of LDL by 4-HNE, a product of lipid peroxidation, has also been shown to aggregate LDL (Hoff *et al.* 1989). However, aggregation of LDL must depend on additional factors, since co-modification of LDL with MDA, another product of lipid peroxidation, can inhibit 4-HNE-mediated LDL aggregation, and aggregation of LDL occurs after oxidation with HOCl,

which causes little lipid peroxidation (Hazell *et al.* 1994). Extensive oxidation of LDL by copper or AAPH, and extensive modification of LDL by 4-HNE have been shown to result in loss of particle integrity. Thus, particles of increased size (Hoff *et al.* 1989; Hoff & O'Neil 1991; Kawabe *et al.* 1994) and lipid vesicles have been observed (Dobrian *et al.* 1993).

Hydrolysis of the cholesterol esters of LDL with fungal cholesterol esterase was shown to produce small lipid droplets and vesicles from LDL (Chao *et al.* 1992; Bhakdi *et al.* 1995). The LDL particles had to be trypsinized before this fungal enzyme was able to act on the particles, but it appeared that trypsinization alone was unable to cause morphological changes in the LDL particles. According to Bhakdi *et al.*, the degree of morphological modification increased still further if the particles were additionally treated with neuraminidase (Bhakdi *et al.* 1995).

Finally, Tertov *et al.* have shown that both glycosylated LDL and desialylated LDL, two forms of LDL found in the circulation, aggregated *in vitro* (Tertov *et al.* 1989; Tertov *et al.* 1992). Interestingly, the same group has provided evidence for a sialyltransferase in blood plasma that can deplete isolated LDL of sialic acid and start a cascade of LDL modifications (Tertov *et al.* 1998).

Effect of extracellular matrix on LDL modification

The ECM of the arterial intima, especially GAGs, can actively participate in the modification of LDL, not only by binding of LDL and various enzymes, but also by modifying LDL and the activity of the enzymes

Modification of the structure of apoB-100 by glycosaminoglycans

Binding of glycosaminoglycans to apoB-100 in LDL has been shown to induce a conformational change in apoB-100 and also changes in the organization of LDL lipids. Thus, binding of glycosaminoglycans to apoB-100 has been shown to increase the microviscosity of the LDL lipids and reduce the thermal stability of the particles (Nakashima *et al.* 1975; Bihari-Varga *et al.* 1981; Camejo *et al.* 1991; Cherchi *et al.* 1994). Moreover, exposure of the polar segments of apoB-100, containing lysine and arginine residues, has been shown to be increased irreversibly (Camejo *et al.* 1991). Consistently, lysine residues of heparin-bound LDL were more readily modified by MDA than of LDL in the fluid phase (Haberland *et al.* 1984). Binding of LDL to GAGs has been shown to alter the pattern of apoB-100 proteolysis by specific proteases (Camejo *et al.* 1991). In addition, oxidation of LDL by copper has been shown to be enhanced after LDL has been released from GAGs (Hurt-Camejo *et al.* 1992). Finally, PLA_2-treated LDL has been shown to undergo aggregation but notably also fusion if the particles have been bound to glycosaminoglycans, an effect that is independent of the degree of phospholipid hydrolysis of LDL (Hakala *et al.* 1999).

Alteration in the rate of LDL modification

A variety of enzymes are able to bind glycosaminoglycans, because they contain "heparin binding sequences". Changes in the rate of LDL modification by GAGs may be due either to enzyme activation/inhibition by GAGs or to alterations in the availability of LDL to the enzyme. As an example of the former alternative, the activity of secretory nonpancreatic phospholipase A_2 against LDL was shown to be increased by CSPG, but decreased by heparin

(Sartipy *et al.* 1996; Sartipy *et al.* 1998). As an example of the second alternative, mast cell chymase, which is bound to the heparin chains of mast cell granules, was shown to hydrolyze preferentially LDL particles also bound to the heparin chains of the granule proteoglycans (Kovanen & Kokkonen 1991).

Aims of the Study

Characteristic of the initiation of atherosclerosis is accumulation of small lipid droplets in the vicinity of collagen fibers, bound to small fibrils tethered to the collagen fibers (Frank & Fogelman 1989). The finding of similar lipid deposits in normal rabbit aorta 2 hours after an intravenous infusion of human LDL showed that these lipid droplets can be formed from human LDL (Nievelstein *et al.* 1991). Kokkonen and Kovanen (1989) demonstrated that similar droplets could also be formed from human LDL on the surface of mast cell granules *in vitro*. This process depended on retention of native LDL by the heparin proteoglycans of the surface of the granules, modification of proteoglycan-bound LDL by proteoglycan-bound protease chymase, and fusion of the modified LDL particles.

On the basis of these findings, experiments were set up to elucidate the mechanisms of formation of the small lipid droplets similar to those associated with collagen in the arterial intima *in vivo*. More specifically, the experiments aimed at studying

i) the factors present in the arterial intima that can explain the preferential accumulation of LDL along collagen fibers (I, II)

ii) the modification(s) of LDL that lead to formation of the small lipid droplets from the LDL particles (III, IV, V)

Methods

Isolation and modifications of lipoproteins

Preparation and labeling of lipoproteins

VLDL, IDL, LDL, and HDL were isolated from plasma of healthy volunteers by sequential ultracentrifugation (Havel *et al.* 1955). Apolipoprotein B-100 of LDL was tritiated by the Bolton-Hunter procedure (Bolton & Hunter 1973) with *N*-succinimidyl[2,3-^{3}H]propionate and iodinated using the iodine monochloride method as described (McFarlane 1958; Bilheimer *et al.* 1972). [^{3}H]cholesteryl linoleate was incorporated into LDL by incubating LDL with solid dispersions of [^{3}H]cholesteryl linoleate on acid-washed Celite 545 in the presence of isolated cholesteryl ester transfer protein (Piha *et al.* 1995).

Fluorescent cholesteryl esters were incorporated into LDL by incubating microemulsions (Via *et al.* 1982) containing 5866 nmol of cholesteryl linoleate, 845 nmol of triolein, 1411 nmol of cholesterol, 1970 nmol of 1-palmitoyl-2-oleoylphosphatidylcholine, and 652 nmol of either Pyr_{10}CE or BODIPY-CE with isolated LDL in the presence of isolated cholesteryl ester transfer protein. The labeled LDL was isolated from the donor microemulsions and CETP by density gradient ultracentrifugation and size-exclusion chromatography. The fluorescently labeled LDL preparations were analyzed for lipid composition and ability to bind to heparin, and no significant changes from native LDL were observed.

Modification of LDL

Lysine and arginine residues of apoB-100 were blocked by treatment of LDL with acetic anhydride (Basu *et al.* 1976) and with 1,2-cyclohexanedione (Mahley *et al.* 1977), respectively. LDL was modified with malondialdehyde (MDA) by incubation with increasing amounts of MDA, freshly prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane, in an ice bath for 3 h (Ylä-Herttuala *et al.* 1989). LDL was glycosylated by incubation in buffer containing 200 mM glucose at 37°C. LDL (1 mg/ml) was proteolyzed by incubation with α-chymotrypsin (0.1 mg/ml) or with trypsin (0.1 mg/ml). LDL was oxidized by copper, by the free radical generating 2,2’-azobis(2-amidinopropane)hydrochloride (AAPH), by hypochlorite (HOCl), and by soybean 15-lipoxygenase (SLO).

Aggregates of LDL were prepared by vortexing a solution of LDL (1 mg/ml) in a conical 10-ml tube at room temperature with a table vortex at full speed. Large and small LDL-antibody complexes were prepared by adding sheep anti-human apoB-100 antibody (Boehringer Mannheim) and goat anti-human apoB-100 antibody (Biodesign International) to LDL, respectively.

Analysis of modified LDL

Biochemical analysis

The charge of LDL was analyzed by electrophoresis on cellulose acetate to measure the degree of acetylation, oxidation, glycosylation, and treatment with 1,2-cyclohexanedione or

MDA. Thiobarbituric acid-reactive substances were measured essentially as described (Hessler *et al.* 1983) to assess the degree of LDL oxidation and modification by MDA. The degree of LDL glycosylation was measured by boronate affinity chromatography (Makino *et al.* 1995). The degree of LDL proteolysis was analyzed by SDS-PAGE on 4-20% gels (Laemmli 1970) and by measuring the amount of trichloroacetic acid (TCA)-soluble apoB-100 radioactivity. The amount of sphingomyelin degraded was measured by thin-layer chromatography (Xu & Tabas 1991). LDL density was measured by equilibrium density gradient ultracentrifugation (Redgrave *et al.* 1975).

Determination of particle size

The degree of LDL aggregation was measured by the turbidity of modified LDL at 680 nm, by the sedimentation of LDL at 14,000*g* for 10 min, and by the ability of LDL to enter 0.5% agarose gel in electrophoresis. The degree of both LDL aggregation and fusion was measured by gel filtration on two Superose 6 HR 10/30 columns connected in series, and by rate zonal flotation of LDL in a linear 7.5-30% gradient of NaBr centrifuged at 285,000*g* for 1 h as described (Polacek *et al.* 1988).

Electron microscopy of LDL particles

To assess small changes in LDL particle size, LDL samples were negatively stained with 1% potassium phosphotungstate, pH 7.4, and viewed and photographed in a JEOL JEM-100CX transmission electron microscope (Forte & Nordhausen 1986). To assess the morphology of the aggregated and fused particles, samples were prepared for thin-section EM. Briefly, LDL samples were cast into 2% agarose gel, fixed with 3% glutaraldehyde, and stained with the osmium/tannic acid/*para*-phenylenediamine technique (Guyton & Klemp 1988). Thin sections were then viewed and photographed in a JEOL JEM-1200EX transmission electron microscope.

Fluorescent resonance energy transfer (RET)

For typical experiments, equal concentrations (50 µg/ml) of Pyr_{10}CE-LDL and BODIPY-CE-LDL were incubated at 37°C in buffer containing 20 µM butylated hydroxytoluene (BHT) in the absence and presence of α-chymotrypsin (10 µg/ml) and PGs/GAGs (10 µg/ml). Fluorescent measurements were performed with a Hitachi F-4000 spectrofluorometer equipped with a thermostated cuvette holder. Excitation and emission wavelengths were set at 346 and 395 nm for direct excitation of pyrene, at 346 and 530 nm for indirect excitation of BODIPY, and at 510 and 530 for direct excitation of BODIPY. RET was expressed as the ratio of indirect to direct excitation of BODIPY.

^{1}H NMR

For most of the NMR experiments, buffer of the LDL samples was changed to 137 mM NaCl, 2.7 mM KCl, 10 µM Na_2EDTA, 10 mM phosphate, pD 7.0 in D_2O by gel filtration twice through PD-10 columns. Measurements were made at 37° on a Bruker DRK 500 MHz spectrometer equipped with a 5-mm NMR tube containing external standard (8 mM 3-trimethylsilyl[2,2,3,3-D_4]propionate) in a sealed coaxial insert. In each experiment, 16-256 free induction decay signals (FIDs) of 64k data points were accumulated, using a pulse repetition time of 6.6 s and 90° pulses. The spectral widths were 6.25 kHz. When H_2O was used in the measurements, the water peak was sup-

pressed by the SHAKA-180° pulse sequence (Hwang & Shaka 1995).

The measured FIDs were Fourier-transformed without apodization to the frequency domain spectra, in which the cholesterol backbone –C(18)$\underline{H}_3$, the terminal methyl –C$\underline{H}_3$ and the methylene =CH-$\underline{CH}_2$-CH= resonances were subjected to lineshape fitting with one, one, and three Lorenzians, respectively. The particles sizes were calculated from the chemical shifts according to Eq. 1, which, in a spherical particle with an isotropic core and a radially oriented surface (Lounila *et al.* 1994), links the frequency of the ith NMR line, v_i, and the lipoprotein particle radius, R,

$$v_i(R) = v_i^0 + \frac{2}{3} v_0 \Delta\chi \ln \frac{R}{R-\Delta} \qquad \text{(Eq. 1)}$$

where v_i^o is the asymptotic value of v_i at limit R à ∞, v_0 is the operating frequency of the spectrometer, $\Delta\chi$ is the anisotropy of the magnetic susceptibility of the particle surface, and Δ is the thickness of the surface. For the calculations, constant values of 2.0 nm for Δ and –0.223 ppm for $\Delta\chi$ were used (Lounila *et al.* 1994).

Isolation and modification of extracellular matrix components

Isolation and modification of decorin from fetal bovine skin

Proteoglycans were isolated from fetal bovine skin essentially as described (Choi *et al.* 1989). PG were extracted with 7.8 M urea, 0.15 M NaCl, 5 mM EDTA, 25 mM Tris, pH 6.6, in the presence of 5 mM ε-aminocaproic acid, 0.2 mM phenylmethylsulfonyl fluoride, and 0.02% (w/v) NaN_3.The mixture was filtered and centrifuged at 100,000*g* for 60 min, the supernatant was batch-adsorbed with CM-Sepharose, and the CM-Sepharose was removed by centrifugation. The supernatant was adsorbed with DEAE-Sepharose, which was packed into a column, and the bound material was eluted with extraction buffer containing 1 M NaCl. The eluted peaks were adjusted to 0.15 M NaCl, loaded on a HiTrap Q column, and eluted with a linear gradient of 0.15-1.0 M NaCl in the extraction buffer. The disaccharides of two proteoglycan preparation were 68% and 63% of dermatan sulfate, 3% and 12% of chondroitin-6-sulfate, and 29% and 25% of chondroitin-4-sulfate. The purity of the preparations was analyzed by N-terminal sequencing, which revealed that the preparations contained >90% of decorin. Decorin core protein was prepared by chondroitinase ABC treatment of the decorin. ^{35}S-Decorin was prepared by labeling the core protein of decorin with ^{35}S-labeling reagent (Bolton & Hunter 1973).

Isolation of proteoglycans from human aorta

Human aortas were obtained at necropsy within 24 h of accidental death. Proteoglycans were isolated from intima-media and purified by ion-exchange chromatography on a Q HiTrap column in 6 M urea, essentially as described previously (Paananen *et al.* 1995; Hurt-Camejo *et al.* 1990). The proteoglycan preparation contained 56% chondroitin-6-sulfate, 25% chondroitin-4-sulfate, and 19% dermatan sulfate.

Isolation of lipoprotein lipase from bovine milk

LPL was isolated from fresh bovine milk by the Intralipid binding method, essentially as described by Posner *et al.* (1983). Briefly, 10 ml of Intralipid (300 mg/ml) was added to 210 ml of

skim milk, incubated for 30 min at 37°, and collected and washed by centrifugation. Thereafter, the floating cakes were delipidated by addition of 40 vol of acetone, filtered through a sintered glass filter covered by another filter (Millipore FH; cat. no. FHUP04700), and washed twice with 40 ml of diethyl ether. The powder was dissolved in 0.75 M NaCl, 0.1% Triton X-100, 20 mM Tris-HCl, pH 7.4, and applied to a heparin column (~30 ml). The column was washed with buffer containing 1 M NaCl, 20 mM Tris-HCl, pH 7.4, and 30% glycerol, and LPL was eluted with buffer containing 2 M NaCl, 20 mM Tris-HCl, pH 7.4, and 30% glycerol. The isolated LPL migrated as a single major band in 4-20% SDS-PAGE gel and, with ^{3}H-triolein as substrate (Saxena *et al.* 1989), was found to have an activity of ~5 mmol oleic acid released/h/mg. LPL was inactivated either by incubation with 1 M GuHCl for 1 h at room temperature or by incubation as dilute solution (10 µg/ml) for 1 h at 37°C.

Interaction of native and modified LDL with extracellular matrix components

Affinity chromatography

Affinity chromatography was performed with a SMART system from Amersham Pharmacia. Binding of LDL to decorin was studied in a 1-ml *N*-hydroxysuccinimidyl ester-activated HiTrap column coupled with 1 mg of decorin. Binding of decorin to LDL was studied in a 1-ml *N*-hydroxysuccinimidyl ester-activated HiTrap column coupled with 1 mg of LDL. Binding of lipoproteins to monomeric and dimeric LPL was studied in a 1-ml HiTrap column coupled with 10 mg of porcine heparin, into which 100 µg of native or monomeric LPL was injected. In some experiments, monomeric LPL was washed from the column with buffer containing 1 M NaCl, leaving dimeric LPL in the column. The components studied were eluted with a linear gradient of NaCl, elution was monitored by UV absorbance at 280 nm, and the NaCl gradient was controlled by measuring the conductance of the eluent. 500-µl fractions were collected and analyzed for radioactivity.

Microtiter well binding assay

Proteins were adsorbed on microtiter wells (Labsystems, Helsinki) by incubation with decorin (10 µg/ml) in PBS at 37° for 18 h, or LPL (10 µg/ml) in PBS containing 10% glycerol at 4°C for 4 h, or fibrillar collagen type I from calf skin (100 µg/ml) in 67 mM phosphate buffer, pH 7.2, at 37°C for 18 h. Nonspecific binding sites in the wells were blocked by incubation at room temperature for 1 h with 250 µl buffer containing the indicated concentration of bovine serum albumin (BSA). Microtiter well assays were performed by incubating the compound to be tested in 50 µl of buffer at room temperature for 1-2 h. BSA-coated wells served as controls for nonspecific binding. Unbound compounds were removed by aspirating the incubation medium, and the wells were then rinsed three times with 250 µl of buffer. The wells were detached and their radioactivities were determined by liquid scintillation counting. The data points indicate the amounts of ligands specifically bound to the compound(s) tested, which were calculated by subtracting the amounts of the ligands bound to the BSA-coated wells from the amounts of the ligands bound to the wells coated with the compound(s) tested.

Gel mobility shift assay

The affinity of LDL for decorin and heparin was measured by the gel mobility shift assay, essentially as described (Camejo *et al.* 1993). In this

assay, fixed trace amounts of ^{35}S-decorin and ^{3}H-heparin were incubated with increasing amounts of LDL in buffer containing 140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.2. Thereafter, the samples were electrophoresed in gels made of Nu-Sieve agarose dissolved in buffer containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. The gels were fixed, dried, and autoradiographed, and the ability of LDL to retard the electrophoretic mobility of decorin and heparin was studied.

Immunohistochemistry

Samples of coronary arteries were obtained from four hearts explanted at cardiac transplantation and snap-frozen in OCT compound; serial frozen sections were then cut at 5 µm. Sections were either fixed with 4% *p*-formaldehyde and stained with Oil Red O or fixed with acetone and prepared for immunohistochemistry. The antibodies used for immunohistochemistry were MB-47 for apoB-100 (dilution 1:100; a kind gift from Dr. J. Witztum, UCSD), MDA2 for malondialdehyde-modified lysine residues (1:100; produced in the Immunology Core Laboratory of the La Jolla SCOR program, a kind gift from Dr. J. Witztum), 5D2 for LPL (1:100; a kind gift from Dr. J. Brunzell, University of Washington), LF-30 for decorin core protein (1:100, a kind gift from Dr. L. Fisher, National Institute of Dental and Craniofacial Research, NIH), MAB1340 for collagen type I (1:10, Chemicon International). Fixed sections stained with standard protocols using the indirect immunoperoxidase method with either HRP-conjugated goat anti-mouse antibody for mouse monoclonal antibodies or biotinylated goat anti-rabbit antibody and HRP-conjugated streptavidin for the rabbit polyclonal antibody. 3-Amino-9-ethylcarbazole was used as chromogen.

Results and Discussion

Association of native and oxidized LDL with collagen type I

Binding of LDL to decorin-coated collagen

When decorin was adsorbed on microtiter wells, increasing the concentrations of LDL, but not HDL_3, was found to lead to binding of increasing amounts of lipoprotein to the decorin (I, Fig. 1). Under physiological ionic conditions, binding of LDL to decorin could be demonstrated in the fluid phase, using a gel mobility shift assay (I, Fig. 3). This interaction was abolished by degradation of the GAG chain of decorin (I, Fig. 4), showing that decorin interacts with LDL through its GAG chain. Finally, the effect of decorin on the interaction between LDL and collagen was studied. Decorin-collagen complexes were allowed to form by incubating decorin with collagen-coated microtiter wells. Examination of the binding of LDL to the decorin-collagen complexes formed showed that native decorin was able to increase the amount of LDL associated with collagen type I by 10-fold, whereas chondroitinase ABC-treated decorin had no effect (I, Fig. 7). Thus, decorin was able to form a bridge between LDL and collagen, by binding to collagen with its core protein, and binding apoB-100 of LDL with its glycosaminoglycan chain (Fig. 5).

Binding of oxLDL to decorin-coated collagen

Preliminary experiments showed that oxidation by copper, hypochlorite, and AAPH increased the binding of oxidized LDL to collagen dose-dependently. The different types of oxidants studied similarly increased the binding of LDL to collagen when the degree of oxidation was similar, as judged by the electrophoretic mobility of LDL. When binding of the same oxidatively modified LDL preparations to immobilized decorin was studied, it was found that, consistently with previous experiments on other types of PGs (Öörni *et al.* 1997), oxidation progressively decreased the amount of LDL bound to decorin. Finally, when collagen was preincubated with decorin, examination of the binding of oxidized LDL to the decorin-coated collagen showed that native decorin progressively inhibited binding of oxLDL to collagen to over 90%, whereas chondroitinase ABC-treated decorin had no effect. Thus, the glycosaminoglycan chains of decorin were able to block the binding of oxidized LDL to collagen, either by competing for binding to the positively charged residues of collagen type I, or by giving the surface of the collagen a strong negative charge that caused repulsion of the oxidized LDL particles.

Effect of lipoprotein lipase (LPL) on the binding of LDL and oxLDL to decorin-coated collagen

When the experiments on microtiter wells were conducted at physiological ionic strength, little binding of native LDL to decorin took place, and no specific binding of copper-oxidized LDL to decorin was detected (II, Fig. 1). However, preincubation of decorin-coated wells with LPL isolated from bovine milk greatly increased the amounts of both native and oxidized LDL bound

to decorin, the dissociation constants being 12 and 5.9 nM, respectively. This increase in binding was found to depend on the binding of the lipoproteins to LPL rather than on modulation of the interaction between decorin and LDL by LPL, since the binding of LDL to decorin-bound LPL and to LPL directly immobilized to microtiter wells were similar in affinity. Finally, the binding of native and oxidized LDL to collagen was tested in the absence and presence of decorin and LPL. The binding of native and oxidized LDL to collagen that had been pre-incubated with decorin was dramatically increased by LPL (23- and 7-fold, respectively), but the presence of LPL had no effect on the binding of native and oxidized LDL to collagen in the absence of decorin (II, Fig. 2). Thus, the ability of collagen to bind lipoproteins was markedly enhanced by lipoprotein lipase, provided that decorin was present on the collagen.

Interaction of lipoproteins with LPL

Experiments on microtiter wells revealed that different preparations of LPL had different abilities to link native and oxidized LDL to decorin. Thus, the amount of oxidized LDL bound to LPL was nearly constant whereas the amount of native LDL bound to LPL differed markedly. Moreover, oxLDL appeared to have a higher affinity for LPL than native LDL. To study these differences, a series of experiments was conducted using affinity chromatography. Preliminary experiments on decorin (affinity) columns revealed that preinjection of LPL retarded elution of native LDL from 50 mM NaCl to ~600 mM NaCl from the column. Thus, it appeared that native LDL could be dissociated from LPL by increasing the ionic strength of the buffer. Copper-oxidized LDL, though it did not bind to the decorin column at all in the absence of LPL, co-eluted from the column with LPL activity at ~800 mM NaCl after pre-injection of LPL. To be able to use higher NaCl concentrations that could potentially also

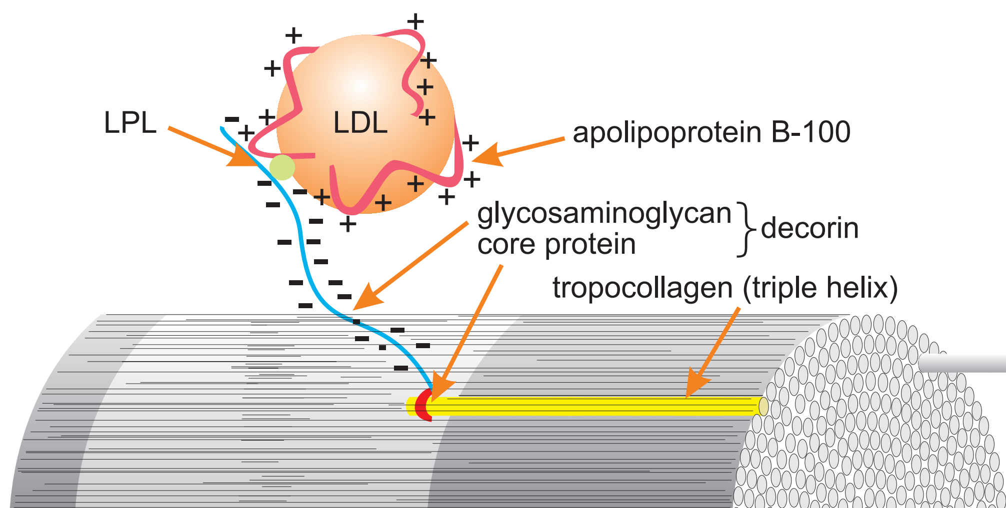

Fig. 5. Schematic picture of the binding of LDL to collagen type I in the presence of decorin and lipoprotein lipase. Adapted from Kovanen & Pentikäinen (1999).

dissociate oxLDL from LPL, affinity chromatography on heparin columns loaded with LPL was also performed. In this system, native LDL was found to elute from the column at ~750 mM NaCl, and VLDL and IDL, which are substrates for LPL, co-eluted from the column at 1.3 M NaCl (II, Fig. 3*A*). When LDL was oxidized with copper, we found that after as little as 2 h the affinity of the LDL for LPL was significantly increased, and at 18 h oxLDL eluted at the leading edge of LPL activity (II, Fig. 3*C*). This was not dependent on the type of LDL oxidation, since similar increases in affinity were also observed with AAPH, HOCl, and SLO-mediated oxidation (II, Fig. 3*C*). Because LPL is known to be present as a catalytically active dimer and a catalytically inactive monomer, it was tested whether the difference between binding of native and oxidized LDL to LPL could be due to binding of the lipoproteins to the different forms of LPL. Mild dissociation of the isolated LPL into inactive monomers was found to have no effect on the binding of native LDL to LPL, whereas it totally abolished the high-affinity binding of oxLDL (II, Fig. 4.). Moreover, when the inactive LPL, which eluted at a lower NaCl concentration than the catalytically active LDL, was removed by washing the column with buffer containing 1 M NaCl, the remaining dimeric LPL had no effect on the binding of native LDL to the heparin column, whereas oxLDL bound to the dimeric LPL with high affinity. This did not depend on the amount of LPL in the heparin column, since similar results were obtained when large amounts (400 µg) of LPL were loaded on the column. Thus, native LDL bound to monomeric LPL, whereas oxidized LDL bound to dimeric LPL. The exact mechanism accounting for this difference in binding could not be resolved, but it appeared that hydrolysis of apoB-100 in oxidation was most likely a contributory factor, since, after extensive proteolytic degradation of apoB-100, LDL was able to bind to dimeric LPL (II, Fig. 5). Moreover, binding to dimeric LPL was found to be mediated by the lipids rather than by apoB-100, as previously suggested, since lipid droplets made of LDL lipids also bound effectively to dimeric LPL (II, Fig. 5). Thus, the differences in the binding of native and oxidized LDL to LPL can be explained at least partly by the binding of the lipoproteins to the two different forms of LPL. The finding that VLDL and IDL preferably bind to dimeric LPL is interesting and most likely important in allowing catalytically active LPL to bind to its substrates.

Ultrastructural localization of LDL, oxidized lysines, lipoprotein lipase, decorin, and collagen type I

To investigate the spatial relationship of the extracellular matrix components studied *in vitro*, samples of coronary arteries were taken from four explanted hearts, and frozen sections were stained immunohistochemically for apoB-100, MDA-lysines, LPL, decorin, and collagen type I. ApoB-100, MDA-lysines, LPL, decorin, and collagen were present in distinct, characteristic, partially overlapping areas of the intima. Thus, apoB-100 was present in all the early lesions (types I-III) in both the superficial proteoglycan-rich and the deep musculoelastic layer of the intima and was concentrated in distinct sectors of the arterial circumference. In advanced lesions (type V), staining for apoB-100 was most intense around the core and shoulder areas of the lesion. Staining for oxidized epitopes (MDA-lysines) was weak or absent in the grossly normal intima, but was clear in lesions of types I-III in the proteoglycan-rich layer, being associated mainly with intracellular lipid deposits. In advanced lesions (type V),

oxidized epitopes were concentrated, like apoB-100, around the core and shoulder areas of the lesion. In early lesions (types I-III), lipoprotein lipase was present subendothelially and in the proteoglycan-rich layer, but notably not in the musculoelastic layer, of the intima. In advanced lesions LPL was found in foam cell-rich areas of the shoulders and sometimes also deeper around the core. Decorin was present in the proteoglycan-rich layer in early lesions (types I-III), and around the core regions in advanced lesions (type V). Staining for collagen type I was weak in areas of thin, grossly normal intima, but in thick intima was clearly present in the proteoglycan-rich layer (lesion types I-III). In advanced lesions, staining for collagen type I was concentrated around the lesion core. Interestingly, we were able to find an early atherosclerotic lesion, characterized by extracellular oil red O-positive material, in which all the components studied were present (II, Fig. 6). Thus, colocalization of LDL with LPL, decorin and collagen type I suggests a role for LPL, decorin, and collagen type I in retention of LDL in the arterial intima.

The present experiments were conducted to find out why LDL appears to be preferentially retained along collagen fibers in the arterial intima. Previous *in vitro* studies on LDL-collagen interaction have used collagen that was isolated and reconstructed *in vitro* and lacked collagen-associated molecules present in the arterial intima. In these studies, binding of native LDL to collagen was poor, but was increased exponentially if the LDL particles were oxidized (Hoover *et al.* 1988; Kalant *et al.* 1991; Kalant & McCormick 1992; Kalant *et al.* 1993; Jimi *et al.* 1994; Greilberger *et al.* 1997). Because decorin has been shown to colocalize with collagen type I in the arterial wall and to bind to collagen *in vitro*, the effect of decorin on the interaction between LDL and collagen was studied. The results show that decorin was able to link native LDL to collagen, whereas binding of oxidized LDL to collagen was effectively inhibited by decorin on collagen. While binding of the core protein of decorin to collagen was evident on microtiter wells in physiological ionic strength, detection of the interaction between the glycosaminoglycan chains of decorin and LDL at physiological ionic strength required sensitive assays, such as the gel mobility shift assay. The decorin for our experiments was isolated from fetal bovine skin and may have differed from human arterial decorin in its ability to bind LDL. However, in affinity chromatography, LDL bound to decorin even more strongly than to a large versican-like proteoglycan isolated from human arterial intimas, implying that decorin from bovine skin was a good model for human arterial decorin.

Is decorin specific in its ability to link LDL to collagen? The arterial intima has been shown to contain a number of different forms of proteoglycan, but only decorin has been shown to be associated with collagen (Riessen *et al.* 1994). Thus, binding of decorin to collagen appears to be specific. LDL was displaced from decorin by several GAGs *in vitro* (I, Fig. 6), suggesting that binding of LDL to decorin is similar to binding of LDL to other GAGs and PGs. However, decorin can potentially bind intermediary molecules both by its GAG chain and by its core protein, which may be differ from that of other proteoglycans, e.g. versican. A study of the effect of decorin on the ability of lipoprotein lipase to enhance binding of LDL to collagen showed that in the absence of decorin, LPL was unable to associate with collagen. However, LPL was able to bind to the GAG

chain of decorin, which allowed association of LPL with collagen. Binding of LPL to decorin is specific for dermatan sulfate, as LPL has been reported not to bind to chondroitin sulfate, the major GAG of versican (Saxena *et al.* 1993a). Interestingly, the ability of LPL to link LDL to decorin and to biglycan were recently compared, and, surprisingly, LPL only enhanced the binding of LDL to decorin (Wagner *et al.* 1997).

The ability of LPL to link lipoproteins to glycosaminoglycans was discovered almost a decade ago (Eisenberg *et al.* 1992; Saxena *et al.* 1992), but binding of lipoproteins to monomeric and to dimeric LPL have not previously been compared. In fact, in many studies in which the structural linking function of LPL has been evaluated, catalytically active LPL has been dissociated into inactive monomers in order to avoid confounding lipolytic activity of the enzyme. These studies have shown that VLDL and LDL bind to the monomeric LPL, but that oxidation decreases the binding of LDL to LPL (Hendriks *et al.* 1996). In human plasma, however, monomeric, catalytically inactive LPL is associated with LDL (Vilella *et al.* 1993), whereas dimeric, catalytically active LPL is associated with triglyceride-rich lipoproteins (Zambon *et al.* 1996), suggesting that the meric state of LPL influences the binding of LPL to lipoproteins. The present experiments showed that native LDL preferentially bound to monomeric LPL, whereas oxLDL, IDL and VLDL preferentially bound to dimeric LPL.

The role of LPL in promoting the development of atherosclerosis in the arterial wall was initially proposed by Zilversmit (1973). Since then, a great deal of indirect evidence has accumulated in support of the proatherosclerotic role of LPL in the arterial wall. Recently, the role of macrophage lipoprotein lipase expression was studied in C57Bl/6 mice transplanted with fetal liver cells from $LPL^{-/-}$, $LPL^{+/-}$, and $LPL^{+/+}$ mice. It was found that lipoprotein lipase expression in the arterial wall promoted the formation of foam cells and the development of atherosclerotic lesions, but it could not be determined from the experiments whether the effect of LPL was structural or enzymatic (Babaev *et al.* 1999). Our immunostaining results in the arterial intima suggest that LPL may be important for the retention of LDL by collagen.

Aggregation and fusion of modified LDL

Ability of the various modifications to trigger fusion of LDL particles

First, we studied the ability of the various modifications of LDL established *in vitro* to cause aggregation and fusion of the particles. A panel of biochemical methods was used for analyzing the formation of enlarged structures 1) to compare the sensitivities and correlation of methods that have been used individually in the literature, 2) to study whether aggregation could be distinguished from fusion by biochemical methods, and 3) to find out whether any particular modification would show a characteristic physicochemical “fingerprint” that could be used for the characterization of LDL isolated from the arterial intima.

As shown in (II), **vortexing** of LDL led to massive aggregation of LDL, which could be demonstrated by the increased turbidity of the sample, by the sedimentation of most of the LDL in low-speed centrifugation, and by the flotation of LDL in rate-zonal centrifugation. Consis-

tently with previous results (Guyton *et al.* 1991), thin-section EM revealed the presence of large lipid droplets, lipid vesicles, and aggregates of native-sized LDL. EM of negatively stained samples showed the presence of massive aggregates, but did not reveal the characteristics of the aggregates. The mean density of the particles was unchanged, but the density distribution was significantly narrowed. Addition of polyclonal sheep-anti **apoB antibody** to LDL that had been developed for nephelometric determination of apoB concentration caused formation of the largest aggregates of the modifications studied. Again, thin-section EM was able to show both the shape and the size of the whole aggregate, and also the presence of only native-sized LDL particles in the aggregates. Similarly, all the biochemical tests showed that most of the LDL particles were present as aggregates. The density of the aggregates was slightly greater than that of LDL, revealing increased protein (antibody) in the aggregates. **Proteolysis** by α-chymotrypsin triggered fusion of the particles with a minimal degree of aggregation of the fused particles. Thin-section EM also showed the presence of membranous material extending from the particles but no true vesicles were observed. Loss of protein decreased the density of the particles to the range of VLDL and IDL lipoproteins. A notable feature was the lack of turbidity and the material sedimenting in low-speed centrifugation, even though other methods showed that most of the LDL particles were increased in size. **Oxidation** by copper led to some degree of aggregation of the particles, but this was not so extensive as was to be expected from previous studies (Hoff & O'Neil 1991; Dobrian *et al.* 1993). After extensive oxidation, particle integrity was lost and fused particles as well as pieces of lipid structures were found by EM. Consistent with previous results by Fong *et al.* (1987), but in contrast to the results of Hoff *et al.* (1992), SDS-PAGE showed aggregated protein larger than apoB-100 and a smear of fragments with no distinct bands. Moreover, the density of the particles had increased significantly. **Lipolysis of the sphingomyelin** of LDL led to the formation of small fused particles as well as particle aggregates, and no individual native-sized particles were present, as judged by gel-filtration chromatography and by rate-zonal flotation. However, the aggregates formed were small, as judged by the lack of turbidity of the samples. Modification of LDL with **MDA** caused a slight increase in the density of the LDL and a small degree of aggregation of native-sized LDL particles, whereas, even after incubation with 200 mM **glucose** for 1 week, aggregation of LDL was minimal.

From the results, it was clear that measurement of turbidity and of sedimentation at low-speed centrifugation revealed only the presence of large aggregates. Gel filtration chromatography, which has traditionally been used to measure particle size, was sensitive in detecting increased particle size, but did not differentiate large aggregates from small aggregates or from fused particles. Moreover, recoveries of samples containing aggregates were poor and their application led to deterioration of the performance of the column. In contrast, rate zonal flotation showed many favorable features in detecting particles of enlarged size. Thus, recovery of the samples, even when extensively aggregated, was good. Moreover, the sensitivity was also fairly good, as judged by the presence of particles floating at an increased rate after modification of LDL by MDA, despite the absence of significant turbidity of the sample. Finally, small aggregates/fused particles could be separated from

larger particles or aggregates, as was shown for example for SMase-treated LDL, which in gel filtration eluted totally in the void volume. Finally, the superiority of thin-section EM over negative staining EM was notable in analyses of aggregated and fused particles.

From this study, it was evident that neither the methods that measure particle size nor those that measure particle density were able to distinguish aggregation from fusion of the particles. Therefore, a more sophisticated methodology was devised. Finally, no physicochemical fingerprint of the modifications was observed, except for a marked increase in the density of oxidized LDL. Therefore, specific chemical or immunological markers of LDL modifications should be used for characterization of LDL isolated from the arterial intima. These include, for example, measurement of ceramide as a marker of SMase action (Schissel *et al.* 1996) and various stable oxidation end-products as markers of lipid peroxidation.

Characterization of proteolytic fusion of LDL by ^{1}H NMR

^{1}H NMR has previously been used for the determination of the lipids in VLDL, IDL, LDL, and HDL on the basis of differences in the magnetic susceptibility of the particles that correlate with the size of the particles. Therefore, experiments were conducted to find out whether ^{1}H NMR could be devised to monitor LDL particle fusion. To be sure that aggregation of LDL without particle fusion does not change the chemical shift of the hydrocarbon ^{1}H NMR spectra, ^{1}H NMR spectra of LDL aggregated with heparin or anti-apoB antibodies were studied. Neither heparin, which bound LDL reversibly, nor goat anti-apoB, which produced aggregates of LDL with a diameter of ~1 μm (i.e. aggregation of hundreds of LDL particles) (IV, Fig. 4), had any effect on the ^{1}H NMR spectra (IV, Fig. 3). But, aggregation of LDL by sheep anti-apoB, which caused formation of massive insoluble aggregates (IV, Fig. 4), caused significant broadening of all lipid resonances, most likely indicating restriction in the tumbling of the large aggregates (IV, Fig. 3). However, the chemical shift of the lipid resonances was unchanged even in the large aggregates, indicating that aggregation does not cause changes in the chemical shift in the ^{1}H NMR spectra of lipid resonances.

With this methodology, the time course of the LDL fusion triggered by α-chymotrypsin was demonstrated. The size of the LDL particles was found to increase exponentially during proteolysis in the spectrometer (IV, Fig. 2). This most likely reflects the necessity for a certain degree of proteolysis of apoB-100 before fusion of LDL begins to take place (Piha *et al.* 1995). Moreover, when the average size of the particles was derived from Eq. 1 during proteolysis, the values obtained correlated well with the values previous obtained with negative staining EM (Piha *et al.* 1995).

Application of the ^{1}H NMR methodology should not be restricted to detection of changes in particle size but should also be used to detect physicochemical changes in the particles. The resonances of the phosphatidylcholine (PC) and sphingomyelin (SM) headgroups have previously been shown to be distinguishable (Murphy *et al.* 1997). Our preliminary results suggest that, during particle fusion, PC and SM behave differently. Thus, the resonance for PC first increased by 10-20% during the initial 3 h and then decreased by 40-60% during 24 h, whereas that of SM gradually decreased by 40-60% after initiation of proteolytic particle fusion. The rela-

tive increase in PC can most likely be explained by release of PC immobilized by apoB-100. The relative decrease in the intensities of both PC and SM, in turn, is likely to reflect the formation of rigid structures from surface lipids of LDL particles in which the mobilities of the PC and SM headgroups are restricted. This is supported by ^{1}H NMR analysis of lipids extracted from proteolyzed lipid particles, which showed that the amounts of PC and SM in the samples were unchanged.

Taken together, ^{1}H NMR appears to be a powerful tool for studies of LDL fusion, especially if structural information about the particles is looked for. One advantage is that it is non-invasive, i.e. no labeling of particles is required, and, in addition, it allows continuous measurements, and gives information on particle structure at the molecular level. However, its drawbacks are the requirement of expensive equipment, and difficult data analysis, and the fact that information on large aggregates cannot be obtained.

Effect of human arterial proteoglycans on proteolytic fusion of LDL

To study the effect of proteoglycans and glycosaminoglycans on the rate of LDL fusion, i.e. on the fusion of LDL in aggregates, a novel type of methodology was devised, based on fluorescent resonance energy transfer (RET). In this system, two different fluorescent cholesteryl ester analogs were incorporated into the cores of different samples of LDL particles with the aid of cholesteryl ester transfer protein (CETP), and LDL fusion was studied in a mixture of the two LDL preparations. The fluorescent probes in the cores of separate LDL particles are, on average, too far apart for RET to occur. However, fusion, though not aggregation, of the particles, allows mixing of the core lipids and RET.

Incubation of LDL in the presence, but not in the absence, of α-chymotrypsin significantly increased RET (V, Fig. 1). The increased RET was limited to particles of increased size, indicating detection of particle fusion (V, Fig. 2). Importantly, the rate of proteolytic fusion of LDL was increased when LDL was complexed with PGs isolated from human arterial intima and with GAGs isolated from the PGs (V, Fig. 3-5). Induction of the rate of particle fusion was correlated both with the ability of the PGs and GAGs to aggregate LDL and with the rate of LDL proteolysis, suggesting that the LDL in the aggregates were more efficiently proteolyzed. Thus, it appears that even small amounts of proteases are able to degrade large amounts of LDL particles when these are aggregated, as in the arterial intima. Moreover, the differences in the ability of GAGs to enhance the rate of proteolytic fusion is interesting in light of the presence increased amounts of specific proteoglycans in distinct regions of atherosclerotic lesions.

The methodology based on RET appeared suitable for studies on LDL fusion. Its strengths are detection of particle fusion even in aggregates with GAGs and continuous monitoring of the process. However, it is laborious (requiring labeling of the LDL particles) and does not give quantitative values for the extent of particle fusion.

The finding that the proteolytic fusion of LDL is enhanced by interaction of LDL with proteoglycans is interesting. Previous work has shown that proteoglycans can induce an irreversible conformational change in apoB-100, which can enhance proteolysis of LDL (Camejo

et al. 1991). Moreover, proteases that interact with GAGs, i.e. have heparin-binding sites, have been shown preferentially to hydrolyze a substrate that is also bound to GAGs (Kovanen 1996). Finally, GAGs could activate a protease by interaction with GAGs. All these possibilities were tested, but no supporting evidence was found for any of the above possibilities. Thus, it appears that, after aggregation by GAGs, which concentrates LDL, proteolysis is more efficient.

General discussion

Characteristic of early atherosclerosis is accumulation of "perifibrous" lipid in the arterial intima (Smith *et al.* 1967). LDL has been shown to accumulate preferentially in the vicinity of collagen fibers, to small fibrils tethered to the collagen fibers (Frank & Fogelman 1989; Nievelstein-Post *et al.* 1994; Tamminen *et al.* 1999) (Fig. 1). The present work has shown that both decorin and lipoprotein lipase may potentially be involved in retaining LDL to collagen. Is retention of LDL to collagen a prerequisite for the development of atherosclerotic lesions? Binding of LDL to proteoglycans, which is one factor in the retention of LDL to collagen, has been shown to be proatherogenic, as mice expressing proteoglycan-binding-deficient LDL were resistant to the development of atherosclerotic lesions despite hypercholesterolemia (Borén *et al.* 1998b). Correspondingly, macrophage LPL expression in the arterial wall, which can also have structural, i.e. LDL-binding, effects, has been shown to be proatherogenic (Babaev *et al.* 1999). Thus, indirect evidence suggested that molecules involved in the retention of LDL in the extracellular matrix promote the development of atherosclerosis.

The present work offers mechanistic clues as to how lipid droplets and vesicles can be derived from modification and subsequent fusion of LDL particles. What is the evidence that the intimal lipid droplets and vesicles are derived from modification of LDL particles? Firstly, similar particles can be found in the intima after intravenous infusion of a large amount of human LDL in normal rabbits (Nievelstein *et al.* 1991) and after incubation of rabbit aortic leaflets with human LDL *in vitro* (Nievelstein-Post *et al.* 1994). Secondly, apoB is colocalized with similar particles, suggesting that the particles are derived from apoB-containing lipoproteins (Mora *et al.* 1987a). Thirdly, the particles are present in the arterial intima before monocytes/macrophages enter the intima (Simionescu *et al.* 1986; Guyton & Klemp 1992), in areas of the arterial intima devoid of macrophages and cell death (Slatter & Smith 1972; Bocan *et al.* 1986; Guyton & Klemp 1993), which makes their cellular origin doubtful. Fourthly, the particles are clearly smaller (diameters 30-400 nm) than intracellular lipid droplets (diameters >400 nm) (Guyton & Klemp 1989) and closer to the sizes of plasma lipoproteins after modifications. Fifthly, their fatty acid composition (rich in linoleate rather than in oleate) also suggests that they are derived from direct deposition of LDL rather than via cellular processing. Taken together, there is a strong case for direct deposition and modification of plasma lipoproteins in the arterial intima. However, alternative mechanisms for the formation of the lipid droplets and vesicles have also been proposed. Thus, it has been suggested that the small lipid droplets and vesicles could be chylomicron remnants that have entered the vascular wall (Mamo & Wheeler 1994; Proctor & Mamo 1998), or lipolytic remnants of VLDL particles, produced either in the arterial intima in the presence of small amounts of HDL or in the plasma by ab-

normal lipolysis (Chung *et al.* 1994). Future work will reveal the relative importance of these various mechanisms.

Finally, what, if any, is the relevance of the small lipid droplets and vesicles to the development of atherosclerotic lesions? Their accumulation in the arterial intima precedes infiltration of monocytes into the arterial intima and coincides with endothelial cell expression of VCAM-1 (Dansky *et al.* 1999), suggesting that the particles could be causing recruitment of monocytes in the arterial intima. Aggregated and fused LDL particles cause foam cell formation *in vitro*, and extracellular lipid droplets and vesicles seemed to disappear from the extracellular matrix as foam cells were formed in the arterial intima (Pasquinelli *et al.* 1989), strongly suggesting that the lipid droplets and vesicles are directly involved in the conversion of macrophages to foam cells *in vivo*. Moreover, the morphology of the lipids in the core of an atheroma closely resembles that of the lipids initially deposited in the subendothelial space (Bocan *et al.* 1986) which have a fatty acid composition compatible with direct deposition of plasma lipoproteins (Smith 1965). Thus, the lipid droplets and vesicles are likely to account for the deposition of significant pro-

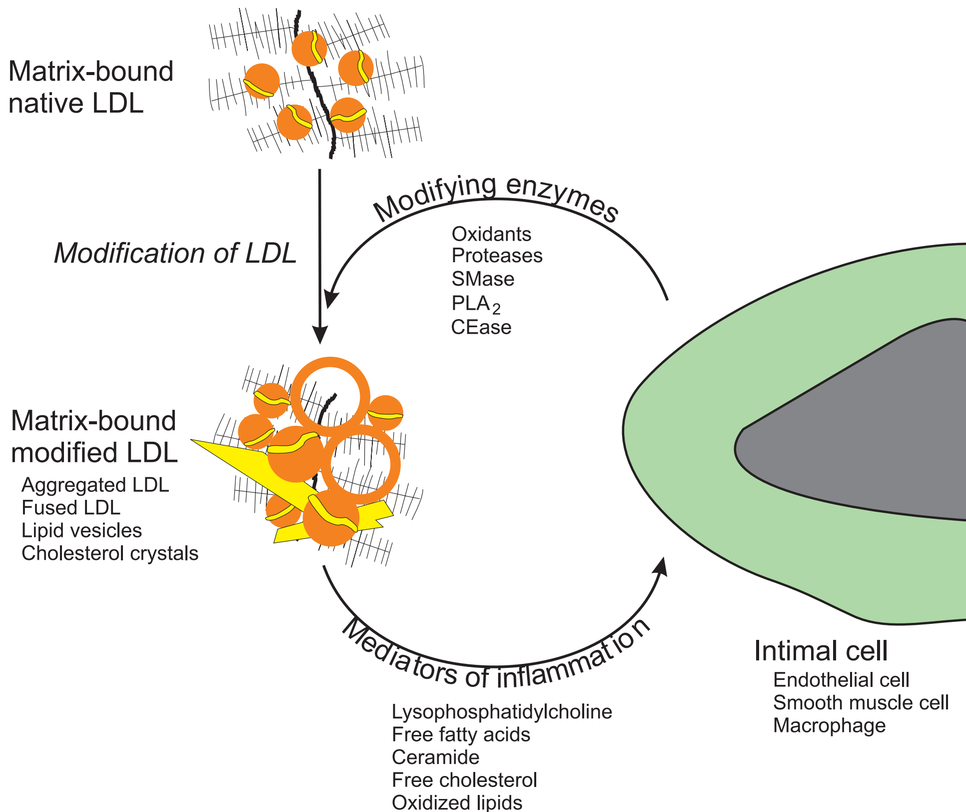

Fig. 6. Vicious circle in the arterial intima. Modification of LDL entrapped in the arterial intima causes formation and release of potent inflammatory mediators. These mediators cause intimal cells to secrete increasing amounts of enzymes capable of modifying LDL and increasing amounts of proteoglycans capable of trapping LDL in the arterial intima. SMase, sphingomyelinase; PLA_2, phospholipase A_2; CEase, cholesterol esterase. From Pentikäinen *et al.* (2000) with permission.

portions of both intra- and extracellular lipids. Finally, modified LDL has recently been shown to have potential proinflammatory properties (Fig. 6). Thus, even minimally oxidized LDL contains modified phospholipids that are chemotactic for monocytes (Berliner *et al.* 1990). Lipolysis of LDL phospholipids by phospholipase A_2 and lipid peroxidation (Steinbrecher *et al.* 1984) generate lysophosphatidylcholine, which has potentially a multitude of pro-inflammatory effects in the arterial intima. Lysophosphatidylcholine and oxidized LDL can be chemoattractants for monocytes and T lymphocytes, induce expression of growth factors and adhesion molecules in endothelial cells, and be mitogenic for macrophages and smooth muscle cells (Hurt-Camejo & Camejo 1997; Steinberg 1997). In addition, oxidized LDL is immunogenic, and autoantibodies are commonly found both in experimental animals and in human subjects with atherosclerotic lesions (Steinberg 1997). Lipolysis of LDL releases free fatty acids, which may lower smooth muscle cell proliferation and induce secretion of ECM capable of trapping increasing amounts of LDL (Olsson *et al.* 1999). Secretory sphingomyelinase can hydrolyse sphingomyelin in modified LDL to ceramide, which, if ingested by intimal cells in large amounts, could escape lysosomal hydrolysis and enters the signaling pathway (Schissel *et al.* 1996). The terminal complement in human atherosclerotic lesions has been shown to be associated with lipid vesicles, and LDL modified *in vitro* into the form of vesicles by cholesteryl esterase has been shown to activate complement by the alternative pathway and also to bind CRP (Bhakdi 1998). Taken together, lipid droplets and vesicles appear not to be innocent by-standers in the arterial wall, but rather participants actively involved in the process of atherosclerosis.

Future perspectives

The advent of modern gene technology including transgenic mice during recent years has changed atherosclerosis research dramatically – the atherosclerotic potential of various proteins can now be assessed *in vivo* by either increasing or decreasing their expression. The problem with the mouse models has been the great differences in lipid metabolism as compared with primates, mice having characteristically low levels of VLDL, IDL, and LDL cholesterol and high levels of HDL cholesterol, and being highly resistant to developing atherosclerotic lesions. Inactivation of the apoE gene has generated hypercholesterolemic mice, with high levels of VLDL cholesterol that develop atherosclerotic plaques similar to those of humans. Importantly, the ultrastructure of the initial extracellular lipid deposits was found to be similar to that previously shown in the cholesterol-fed rabbit, the WHHL rabbit, and rabbits injected with human LDL (Frank & Fogelman 1989; Nievelstein *et al.* 1991; Tamminen *et al.* 1999). Using similar models, it remains an interesting task for the future to evaluate the atherosclerotic potential of the various proteins characterized in the present study that are suggested to be involved in the retention and accumulation of LDL. So far, macrophage expression of LPL in the arterial wall has shown to promote the development of atherosclerotic lesions in cholesterol-fed mice (Babaev *et al.* 1999).

Conclusions

1. Decorin and LPL, two components of the extracellular matrix present in the arterial intima, significantly influence the interaction between lipoproteins and collagen, the former allowing binding of native, but not oxidized, LDL to collagen and the latter mediating high-affinity binding of both native and oxidized LDL to decorin-coated collagen. Immunohistochemical study of the human arterial intima suggested that these interactions are of potential significance in LDL retention in the arterial intima.

2. Monomeric, *i.e.*, catalytically inactive, and dimeric, *i.e.*, catalytically active, forms of LPL interact differently with lipoproteins, the former preferably binding native LDL and the latter VLDL, IDL, and oxLDL. This finding may be important in understanding lipoprotein physiology in the circulation, targeting catalytically active lipase to its substrates VLDL and IDL, and catalytically inactive lipase to LDL, which can be effectively removed by the liver. This finding is supported by previous studies showing association of catalytically inactive LPL with LDL and of catalytically active LPL with lipoproteins larger than LDL in human plasma. The preferential binding of oxLDL to dimeric LPL and of native LDL to monomeric LPL, in turn, may have pathophysiological implications for the arterial wall. Both monomeric and dimeric LPL have been found in a number of the tissues studied. Whether native and oxLDL can be targeted differently by the LPL in the arterial intima remains to be studied.

3. Particle fusion depends on extensive modification of the surface of LDL particles. Of a set of established LDL modifications studied, only those that caused a significant surface defect in the particles, i.e. proteolysis, oxidation, lipolysis of sphingomyelin, and the strong mechanical force induced by vortexing, caused fusion of the particles.

4. Proteolytic fusion of LDL particles was enhanced by proteoglycans isolated from the human arterial intima. This effect was found to correlate both with the ability of the proteoglycans and glycosaminoglycans to form complexes with LDL and with the rate of LDL proteolysis. Taken the presence of increased amounts of specific proteoglycans in atherosclerotic lesions this finding may be of potential pathophysiological significance.

5. The novel methodology comprized application of ^{1}H NMR and fluorescent resonance energy transfer, allowing specific detection of particle fusion, and it is noteworthy that the fluorescent resonance energy transfer methodology was not disturbed by particle aggregation. Information of this kind opens up new possibilities for further studies on LDL particle fusion, the key event in the accumulation of extracellular lipid.

6. The work has provided mechanistic clues to the retention of LDL by collagen and the formation of lipid droplets from LDL. A conceptually novel aspect is the taking into account of the presence of collagen-associated molecules in the interaction between LDL and collagen. Decorin may be important not only in the direct binding of LDL, but also in the binding of such molecules as LPL, which structurally enhance the binding of LDL to collagen, and enzymes like phospholipase A_2, which cause aggregation and fusion of the bound LDL.

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