Review

Metabolism and Modification of Apolipoprotein B-Containing Lipoproteins Involved in Dyslipidemia and Atherosclerosis

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Increased levels of apolipoprotein B (apoB)-containing lipoproteins, such as low density lipoproteins (LDL) and chylomicron remnants, are associated with the development of atherosclerosis. Chylomicrons containing apoB-48 are secreted from the intestine during the postprandial state, whereas very low density lipoproteins (VLDL) containing apoB-100 are constitutively formed in the liver. Chylomicron remnants and VLDL remnants are produced by the lipoprotein lipase-mediated lipolysis of triglycerides, which is activated by apolipoprotein C-II bound on the particle surfaces. The hepatic uptake of these remnants is facilitated by apolipoprotein E (apoE), but is inhibited by apolipoproteins C-I, C-II and C-III. In the plasma, VLDL remnants are further converted into LDL by the hydrolysis of triglycerides. ApoB-100 is responsible for the hepatic uptake of LDL. LDL receptor, LDL receptor-related protein and heparan sulfate proteoglycans are involved in the hepatic clearance of lipoproteins containing apoB-100 and/or apoE. The subendothelial retention and modification of apoB-containing lipoproteins are crucial events in the initiation of atherosclerosis. In the subendothelium, the uptake of modified lipoproteins by macrophages leads to the formation of foam cells storing excess amounts of cholesteryl esters and subsequently to apoptosis. This review describes the current knowledge about the metabolism and modification of apoB-containing lipoproteins involved in dyslipidemia and atherogenesis. In particular, I focus on the effects of apolipoproteins, lipid composition and particle size on lipoprotein metabolism and on the roles of cholesterol, sphingomyelinase and apoB denaturation in macrophage foam cell formation and apoptosis. A detailed understanding of these mechanisms will help to develop new therapeutic strategies.

Key words lipoprotein; apolipoprotein; cholesterol; sphingomyelinase; foam cell formation; apoptosis

1. INTRODUCTION

Plasma lipoproteins consist of a hydrophobic core containing triglycerides (TG) and cholesteryl esters (CE), a surface monolayer containing phospholipids (PL) and unesterified (free) cholesterol (FC), and surface-bound apolipoproteins (Fig. 1A). The main PL components of lipoproteins are phosphatidylcholine (PC) and sphingomyelin (SM). The lipoprotein core also contains small amounts of lipid-soluble vitamins. Based on the densities determined by the relative contents of lipids and proteins, lipoproteins are classified into chylomicrons (CM) (<0.94 g/mL), very low density lipoproteins (VLDL) (0.94 – 1.006 g/mL), intermediate density lipoproteins (IDL) (1.006 – 1.019 g/mL), low density lipoproteins (LDL) (1.019 – 1.063 g/mL), and high density lipoproteins (HDL) (1.063 – 1.210 g/mL).¹⁾ CM is very large spherical particles (75–450 nm diameter) containing only one molecule of apoB-48. CM is formed in the intestine and transports dietary TG and cholesterol to the liver and peripheral tissues (Fig. 2). On the other hand, VLDL (60–80 nm diameter) is synthesized in the liver and contains a single copy of apoB-100. In the plasma, CM remnants and IDL, also called VLDL remnants, are formed by the lipoprotein lipase (LPL)-mediated lipolysis of TG. Although VLDL remnants are partially cleared by the liver, a large part of VLDL remnants are converted into LDL by further hydrolysis of TG. Thus, LDL (18–25 nm diameter) also contains a single copy of apoB-100.²⁾ These apoB-containing lipoproteins also bind the exchangeable apolipoproteins, such as apolipoprotein A-I (apoA-I), apolipoprotein C-I

(apoC-I), apolipoprotein C-II (apoC-II), apolipoprotein C-III (apoC-III), and apolipoprotein E (apoE). On the other hand, HDL (7.2–12 nm diameter) is formed from peripheral tissues, and has mainly apoA-I but no apoB.³⁾ The apolipoprotein composition is characteristic of each lipoprotein class.

A high level of plasma LDL is a strong risk factor for atherosclerosis, while a high level of HDL is protective against atherosclerosis. There are a positive correlation between LDL-cholesterol levels and coronary heart disease (CHD) and an inverse relationship between HDL-cholesterol levels and CHD.^{4,5)} Plasma TG level is a significant predictor of CHD, but is weaker as an independent risk factor than LDL-cholesterol level or HDL-cholesterol level.⁶⁾ Statin treatment reduces coronary events and improves survival, which is attributed to the reduction in LDL-cholesterol.^{4,5)} CM remnants are also known to be atherogenic.⁷⁾ Patients with postprandial hyperlipidemia have premature clinical signs of atherosclerosis.^{8,9)} In addition, the levels of lipoprotein (a) (Lp(a)) are strongly connected with increased risk of atherosclerotic vascular disease.¹⁰⁾ The level of small dense LDL (sdLDL) cholesterol is a significant independent determinant of CHD risk.¹¹⁾

Lipid emulsion particles have been used as models for plasma lipoproteins. However, the lipid emulsions may not necessarily represent the nature of lipoproteins owing notably to the lack of apoB. ApoB plays a crucial role in the maintenance of the lipoprotein structure, but cannot be properly reconstituted on emulsion particles at present. However, lipid emulsions are useful to elucidate the mechanisms of lipoprotein metabolism, because they enable easy control of the lipid and protein con-

stituents and the size of the particles.

2. METABOLISM OF APOB-CONTAINING LIPOPROTEINS

2.1. Lipoprotein Assembly In humans, apoB-48 is used for CM assembly in intestinal enterocytes during the postprandial state, whereas apoB-100 is used for the constitutive formation of VLDL in hepatocytes (Fig. 2). It has been proposed that intestinal and hepatic lipoprotein assembly begins with the synthesis of primordial lipoproteins.^{12–14)} This step involves the release of apoB with lipids derived from the endoplasmic reticulum (ER) membrane to the ER lumen, and is critically dependent on the lipid transfer activity of microsomal triglyceride transfer protein (MTP). Proper folding and stability of apoB requires the MTP-dependent transfer of both polar and neutral lipids from the ER membrane or some other donor site to nascent apoB during translation. The primordial lipoproteins are relatively small dense particles with a maximum diameter of approximately 25 nm and consist of apoB associated with PL monolayer with some TG. In addition, progressively larger TG-rich lipid droplets of different sizes are formed in the ER lumen, and their synthesis occurs independent of apoB synthesis. MTP transfers TG to these droplets, and facilitates their formation. The TG-rich lipid droplets are assumed to fuse with primordial lipoproteins in a process called core expansion. This step may be crucial in rendering these droplets secretion-competent and may be the rate-limiting step that determines the transport of TG from ER to the Golgi complex. These partially matured lipoproteins are delivered to the Golgi for final processing prior to secretion.¹⁵⁾ The transport of TG-rich particles out of the ER to the Golgi complex is facilitated by the acquisition of apoB. ApoB-48, apoA-I, apolipoprotein A-IV (apoA-IV), apoCs are associated with nascent CM.

In abetalipoproteinemia, deficiency in intestinal lipoprotein assembly and secretion results in malabsorption of dietary fat and fat-soluble vitamins.¹⁶⁾ Patients are characterized by severe fat malabsorption and TG accumulation in enterocytes. The synthesis of intestinal CM and liver VLDL is absent in abetalipoproteinemia due to a defect in the MTP activity.^{17,18)} MTP is an ER-localized heterodimeric protein, which is composed of a 97-kDa subunit complexed with the ubiquitous ER-folding enzyme protein disulfide isomerase.¹⁹⁾ The 97 kDa MTP subunit is necessary for lipid transfer activity. The protein disulfide isomerase subunit containing an ER retention

signal is important to retain the MTP subunit in the lumen of the ER. Expression of apoB without MTP leads to the intracellular degradation of apoB.

In CM retention disease, the assembly and secretion of apoB-containing lipoproteins is impaired only in the intestine but not in the liver.^{20,21)} CM retention disease is characterized by diarrhea with steatorrhea, typical lipid-filled enterocytes, and low plasma cholesterol levels. However, apoB-48 synthesis and MTP levels appear normal in the intestine.^{18,22)} CM retention disease is a specific defect of intestinal lipoprotein secretion. The *SAR1B* gene is responsible for CM retention disease and encodes the Sar1b protein, which is involved in the CM transport from the ER to the Golgi apparatus.²³⁾

Mipomersen, an apoB antisense oligonucleotide, reduces LDL cholesterol, apoB, and Lp(a) in both homozygous and heterozygous familial hypercholesterolemia patients.^{24,25)} The administration of lomitapide, a MTP inhibitor, results in reductions of LDL cholesterol, apoB and TG and has no significant effect on Lp(a).²⁶⁾

2.2. Lipolysis of Lipoproteins Nascent CM secreted into the lymphatic system enter the circulation *via* the thoracic duct.⁷⁾ The heart and lung are the first organs encountering postprandial CM, and these two organs are the sites of the robust expression of LPL (Fig. 2). LPL is associated with the luminal side of capillaries and arteries, and hydrolyzes TG to produce free fatty acids. The hydrolysis results in the delivery of free fatty acids to peripheral tissues. LPL converts CM to remnants and begins the cascade required for the conversion of VLDL to LDL. Some LPL dissociates from endothelial cells during lipolysis and continues to hydrolyze lipoprotein TG in the bloodstream.²⁷⁾ CM TG clearance is much faster than VLDL TG clearance.^{27,28)} Larger VLDL particles are removed from the bloodstream before their conversion to LDL, whereas more small VLDL particles are converted to LDL.²⁹⁾ Smaller, but not larger, VLDL particles are also substrates for hepatic triglyceride lipase (HL).²⁷⁾ Plasma LPL is correlated with plasma free fatty acid levels.³⁰⁾ ApoC-II present in CM and VLDL acts as the activator of LPL. Newly formed CM that is isolated from the lymph before entry into the circulation contains very little apoC-II.³¹⁾ There is an exchange of apoCs and apoE on HDL for apoA-I and apoA-IV on CM.²⁷⁾

Cholesteryl ester transfer protein (CETP), a plasma protein of 476 amino acid residues, catalyzes non-directional equimolar exchange of nonpolar lipids, CE and TG, among lipoprotein subfractions.³²⁾ CE is actively generated in plasma HDL

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Fig. 1. Schematic Model of Lipoprotein Particle

(A) A lipoprotein particle is composed of a hydrophobic core containing TG and CE and a surface monolayer containing PL, mainly PC and SM, and FC. Apolipoproteins are bound on the particle surface. (B) At the lipoprotein surface, SM molecules increase the acyl chain order and decrease the head group hydration, whereas FC molecules increase the acyl chain order and head group hydration.

Fig. 2. Metabolism of ApoB-Containing Lipoproteins

CM with apoB-48 and VLDL with apoB-100 are secreted from the intestine and liver, respectively. In the bloodstream, CM remnants are formed by the LPL-mediated lipolysis, which is activated by apoC-II. The lipolysis converts VLDL to VLDL remnants and subsequently to LDL. LDL is taken up into the hepatocytes by LDLR. ApoE-enriched lipoprotein remnants are internalized by the hepatocytes through LDLR or HSPG-LRP pathway. ApoCs prevent the apoE-mediated hepatic uptake of lipoprotein remnants.

Fig. 3. Development of Atherosclerosis Associated with Modified Lipoproteins

Atherosclerosis is initiated by the subendothelial retention of apoB-containing lipoproteins. In the subendothelium, apoB-containing lipoproteins bind to PGs. LPL promotes the binding of lipoproteins to PGs. These lipoproteins are modified by oxidizing agents and enzymes, SMase, phospholipases, LPL and proteases, and are subsequently aggregated. In addition to apoB, these lipoproteins contain apoE. Monocytes enter the subendothelium and then differentiate into macrophages. The uptake of modified lipoproteins by macrophages results in the formation of foam cells storing excess amounts of CE. Furthermore, the accumulation of modified lipoproteins induces the apoptosis of macrophages.

Fig. 4. Formation of Ceramide-Enriched Microdomains in Emulsion Particles

(A) Phase separation in emulsion particles visualized by confocal microscopy. TO-PC, TO-PC/SM and TO-PC/ceramide emulsions contained two fluorescent probes, BODIPY-PC (green) and DiI-C₁₈ (red). BODIPY-PC favors the fluid phase, whereas DiI-C₁₈ predominantly partitions into the ordered membrane phases. In TO-PC and TO-PC/SM emulsion particles, the uniform distributions of BODIPY-PC in the surface and DiI-C₁₈ in the surface and core were observed. In TO-PC/ceramide emulsion particles, two fluorescent probes were non-uniformly distributed. DiI-C₁₈ fluorescence identified the ceramide-enriched microdomains. Ceramide molecules formed three-dimensional, but not in-plane, microdomains extending from core to surface of the particles. The treatment of TO-PC/SM emulsions with SMase led to the formation of ceramide-enriched microdomains and to the fusion of emulsion particles. These images are reproduced from ref. 288. (B) Schematic diagram of ceramide-containing emulsion particles. SMase hydrolyzes SM to ceramide and induces the formation of ceramide-enriched microdomains.

by lecithin:cholesterol acyltransferase (LCAT). Consequently, CETP mediates the net movement of CE from HDL to VLDL and CM and that of TG from VLDL and CM to HDL.³²⁾ Therefore, LPL activity regulates HDL cholesterol levels by decreasing plasma TG.²⁷⁾ LPL activity is correlated with HDL levels.³³⁾ TG-containing HDL are better substrates for HL. Unlike LPL, HL preferentially hydrolyzes lipoprotein PL but also has substantial TG lipase activity.^{34,35)} HL is synthesized primarily in hepatocytes and is bound mostly to hepatic and endothelial heparan sulfate proteoglycans (HSPGs) in the hepatic sinusoids.³⁶⁾

Glycosylphosphatidylinositol-anchored HDL-binding protein-1 (GPIHBP1) is expressed on the luminal side of the endothelium, binds circulating LPL and TG-rich lipoproteins, and promotes lipolysis.³⁷⁾ Mice lacking GPIHBP1 manifest severe chylomicronemia as a result of defective lipolysis and develop progressive aortic atherosclerosis.^{38,39)} In addition, a homozygous missense mutation in *GPIHBP1* causes chylomicronemia in humans.⁴⁰⁾

2.3. Hepatic Uptake of Lipoproteins The space between the endothelium and hepatocytes is called the space of Disse, which is rich in HSPGs and contains an abundance of apoE secreted by hepatocytes.¹⁾ CM remnants enter the space of Disse through the fenestrated sinusoidal endothelium, which acts as a filter that limits the entry of large CM.⁴¹⁾ After entering the space of Disse, lipoprotein remnants become enriched in apoE. The increased amount of apoE enhances the binding of the remnants to HSPGs on the surface of hepatocytes, followed by internalization of the particles through either LDL receptor (LDLR) or LDL receptor-related protein (LRP)^{1,42)} (Fig. 2). HSPGs play a major role in the rapid sequestration of the remnants within the space of Disse, with apoE, HL and LPL serving as ligands.¹⁾ HSPGs act as a reservoir for apoE, which is a critical ligand for the clearance of lipoprotein remnants. The ligand-binding region is not present on apoB-48. LPL bound on the remnant particles is carried into the space of Disse.⁴³⁾ About half of the VLDL remnants are taken up by the liver through apoE-mediated pathways, and the remainder are converted to LDL containing only apoB-100.¹⁾ ApoB-100-containing lipoproteins are removed from the plasma by LDLR. The liver is the primary site of LDL degradation. LDL particles are generally free of apoE and have lower affinity for LDLR.³⁴⁾

Even in the absence of LDLR, the sequestration of the remnants occurs, followed by slow endocytosis *via* the HSPG-LRP pathway.⁴⁴⁾ On the other hand, the absence of the LRP is fully compensated for by the upregulation of LDLR in the liver.⁴⁵⁾ Therefore, under normal physiological conditions, LDLR and LRP participate in the internalization of remnant lipoproteins in the liver. However, in the absence of apoE, the sequestration and endocytosis of remnants are extremely restricted.

ApoCs inhibit the apoE-mediated hepatic uptake through the displacement of apoE on these particles or through a direct interaction with apoE. The hepatic uptake of remnant particles is governed by the balance of apoE and apoCs on the particle surface.⁴⁶⁾

3. ATHEROSCLEROSIS

3.1. Subendothelial Retention and Modification of Li-

poproteins Atherosclerosis is a multifactorial disorder, and involves many processes, such as lipoprotein retention, lipoprotein modification and aggregation, endothelial alteration, macrophage chemotaxis and foam cell formation, and smooth muscle cell migration and alteration.

Atherosclerosis is initiated by the subendothelial retention of apoB-containing lipoproteins including LDL, lipoprotein remnants, and Lp(a)^{47–50)} (Fig. 3). The subendothelial deposition of cholesterol is correlated with the level of arterial exposure to cholesterol-rich lipoproteins.⁴⁷⁾ Even in the absence of other risk factors, elevated levels of lipoproteins containing apoB can drive the development of atherosclerosis in humans and animals.⁵¹⁾ LDL-lowering drugs are the most effective therapy against atherothrombotic cardiovascular disease and decrease the probability that apoB-containing lipoproteins will be retained in the subendothelium.⁴⁹⁾

Approximately 85% of arterial lipoprotein delivery to the subendothelium is estimated to occur *via* transcytosis, whereas lesser amounts of lipoproteins are delivered *via* gap junctions or other processes.⁵²⁾ ApoB-containing lipoproteins greater than 70 nm cannot traverse the endothelium because of the size limitation of transcytotic vesicles.^{47,53)}

The dense extracellular matrix of the subendothelium forms an organized tight network and plays a major role in the retention of atherogenic lipoproteins. Subendothelial matrix molecules exist in the extracellular space and on the cell surface in the intima, and mainly consist of proteoglycans (PGs), collagen, and elastin.⁵⁴⁾ The three major classes of glycosaminoglycans (GAGs) in the vascular system are heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS).⁵⁵⁾ In particular, PGs containing side chains of CS play a major role in the retention of atherogenic lipoproteins. The interaction between atherogenic lipoproteins and PGs involves an ionic interaction between basic amino acids in apoB or apoE and negatively charged sulfate groups associated with extracellular and cell surface PGs.^{48,49,54,56,57)} Lipoprotein lipids and PG core proteins also participate in the interaction.^{49,57)} Among CS-containing PGs, biglycan and versican are especially important in the apoB-containing lipoprotein retention within arteries.^{49,57)} In addition, LPL facilitates the binding of apoB-containing lipoproteins to PGs by acting as a bridging molecule.^{47,49,54)} In the extracellular matrix of the arterial intima, LPL is secreted by the intimal macrophages and smooth muscle cells.^{58,59)}

In the process of atherogenesis, lipoprotein particles are modified and aggregated in the arterial walls. These modifications to lipoproteins include lipolysis by LPL, sphingomyelinase (SMase) and phospholipases, oxidation with various agents, proteolysis, glycosylation, desialylation, and complexation with PGs.^{49,54,60)} Aggregated lipoproteins have an increased binding affinity for PGs, and their larger size physically prevents their release from the subendothelial space.^{53,61)} SMase induces both aggregation and fusion of lipoprotein particles, but phospholipase A₂ induces only aggregation of the particles.⁶¹⁾ Oxidation of lipoproteins involves the attack on many constituents, including cholesterol, fatty acids, antioxidants and apoB.⁶²⁾ Lipoproteins isolated from lesions contain lipid oxidation products, ranging from relatively early products, such as cholesteryl linoleate hydroperoxide, hydroxide and ketone, and isoprostanes, to advanced short-chain aldehydes esterified to CE or PL.⁶²⁾ Oxysterols are also elevated

in the plaque tissues.⁶²⁾ Cathepsins S, K and F are present in the arterial intima and are capable of degrading apoB, which results in enhanced retention of lipoproteins to arterial PGs.⁶³⁾

Another important lipoprotein in lesion development is Lp(a). Lp(a) is a form of LDL that is modified in the liver by covalent attachment of apoB to apo(a), a member of the plasminogen gene family. In the subendothelial matrix, fibronectin binds Lp(a) *via* the heparin binding domain of fibronectin and the lysine binding sites of apo(a).⁶⁴⁾ In addition to LDL, Lp(a) is retained in the arterial intima and is modified by the intimal enzymes and agents.⁵⁴⁾ Lp(a) is rich in potentially atherogenic oxidized PL.⁶⁵⁾ Lp(a) also has antifibrinolytic effects, which enhances its atherogenicity.⁶⁶⁾

The size of human LDL can be separated into two phenotypes: large buoyant LDL (1.019–1.043 g/mL) and sdLDL (1.044–1.063 g/mL). SdLDL is characterized as 18.0–20.5 nm in diameter.¹¹⁾ SdLDL is more atherogenic than large buoyant LDL. SdLDL particles have higher affinity for PGs than large buoyant LDL particles.⁶⁷⁾

3.2. Foam Cell Formation The perturbation of the arterial endothelium leads to the upregulation of cell adhesion molecule.⁶⁸⁾ The blood-borne monocytes enter susceptible areas of the subendothelium of large and medium-sized arteries and then differentiate into macrophages, which is one of the key cellular events during atherogenesis⁶⁹⁾ (Fig. 3). Smooth muscle cells also migrate into the intima.⁷⁰⁾ The lipoprotein modification in the arterial wall leads to the macrophage chemotaxis.⁷¹⁾

The lesions are initiated by the formation of fatty streaks in the artery, when macrophages in the vessel wall take up lipoproteins from the subendothelial space.⁷²⁾ Accumulation of excess cholesterol derived from lipoproteins in arterial macrophages is one of the critical steps in the progression of atherosclerosis. The uptake of matrix-retained lipoproteins by macrophages and smooth muscle cells leads to the formation of foam cells storing a large amount of CE.^{50,70,73)} However, native LDL is incapable of generating foam cells from macrophages.⁷⁴⁾

The uptake of modified lipoproteins by macrophages is a complex process involving receptor-mediated endocytosis and phagocytosis.^{75,76)} Macrophages express LDLR as well as LRP. In addition, the apoB-48 receptor on macrophages is implicated in the apoE-independent pathway for the uptake of TG-rich lipoproteins and recognizes apoB-48 and apoB-100.⁷⁷⁾ Furthermore, macrophages express several different scavenger receptors that recognize modified lipoproteins.^{62,78)} Aggregated lipoproteins are taken up *via* phagocytosis, by LRP, by scavenger receptors, and through pinocytosis.^{75,79–81)} In pinocytosis, the aggregated LDL induces and enters surface-connected compartments.⁸⁰⁾ Oxidized LDL (oxLDL) is a ligand for scavenger receptors expressed on the macrophage surface, including class A scavenger receptor type I/II and CD36.^{62,78)} These scavenger receptors are multifunctional and interact with several structurally different ligands.^{62,78)}

LDL with native apoB-100 enters macrophages by LDLR and is a poor inducer of foam cell formation.⁷⁴⁾ After LDL particles are taken up by macrophages and delivered to the lysosomes, CE is hydrolyzed to FC by lysosomal acid lipase. The resulting FC is transferred to the ER and reesterified by acyl-CoA:cholesterol acyltransferase (ACAT), which is localized in the ER membranes.⁸²⁾ Niemann–Pick C1 (NPC1) pro-

tein on the lysosomal membranes accepts FC from Niemann–Pick C2 (NPC2), a soluble luminal protein of lysosomes, and then mediates FC trafficking to the ER membranes or plasma membranes, depending on the vesicular transport or oxysterol-binding protein-related proteins.^{83,84)} In the hydrophobic hand-off model, the patches of amino acids on NPC1 and NPC2 interact so that the transfer of FC from NPC2 to NPC1 is achieved.^{83,84)}

Lysosomal FC predominantly distributes to the plasma membrane, where FC is available for the efflux mediated by ATP-binding cassette transporter A1 (ABCA1) and apoA-I leading to the formation of HDL. After reaching a threshold level, the cellular FC is esterified by ACAT, which leads to the accumulation of intracellular CE.⁸¹⁾ CE accumulates in cytoplasmic droplets during the early fatty streak stage of atherosclerosis.⁶²⁾ However, CE also increasingly accumulates within large swollen lysosomes of foam cells in the fibrous plaque.⁶²⁾

3.3. Apoptosis of Macrophages Numerous studies have identified apoptosis as a prominent feature of atherosclerosis.^{85,86)} All cell types present in atherosclerotic plaques, including endothelial cells, smooth muscle cells, lymphocytes and macrophages, undergo apoptosis. The accumulation of modified lipoproteins induces the apoptosis of monocyte-derived macrophages^{85–87)} (Fig. 3). In early lesions, where phagocytic clearance of apoptotic cells appears to be efficient, macrophage apoptosis may decrease macrophage burden and slows lesion progression.^{86,88,89)} On the other hand, macrophage apoptosis in late lesions causes necrotic core formation, which promotes inflammation, plaque rupture, and thrombosis.⁸⁶⁾ The membranes of the apoptotic cells are eventually disrupted, leading to the release of intracellular contents and to the inflammatory responses.⁸⁶⁾ Therefore, the macrophage apoptosis is a crucial determinant of lesion development.

Macrophages in advanced atherosclerotic lesions accumulate large amounts of FC, which is a potent inducer of apoptosis.^{82,86,87)} Macrophages have several mechanisms to prevent the accumulation of excess FC, such as cholesterol esterification by ACAT, efflux of cellular FC by ABCA1, and down-regulation of LDLR and cholesterol biosynthetic enzymes.⁸²⁾ In atherosclerotic lesions, however, these mechanisms are often ineffective, which results in the accumulation of excess FC in macrophages. High FC/PL ratio in cellular membranes alters the functions of integral membrane proteins due to high membrane rigidity.⁸²⁾ The PL synthesis also increases to offset the harmful effects of excess FC, and, as a consequence, the PL-containing whorl-like membrane structures exist in the lesional macrophages.⁸²⁾

The abnormal enrichment of the plasma membrane with FC results in the dysfunction of membrane-associated enzymes and transporters and in subsequent macrophage death.⁹⁰⁾ On the other hand, the FC accumulation in the ER membrane induces the unfolded protein response, leading to an apoptotic response in the macrophages.⁹¹⁾ The ER membrane is normally FC poor and highly fluid.⁸⁷⁾ Needle-shaped cholesterol crystals typically form in the extracellular regions of advanced atherosclerotic lesions.⁸²⁾ Intracellular cholesterol crystals are also present in the atherosclerotic lesions and physically disrupt the intracellular structures.^{82,92)} In addition, excess intracellular FC accumulation promotes the generation of cytotoxic oxysterols.⁸²⁾ The macrophage apoptosis induced by oxLDL is

indeed mediated by oxysterols.⁹³⁾ Oxysterols induce apoptosis through various mechanisms including lysosomal rupture, elevation in intracellular calcium, and the mitochondrial apoptotic pathway.⁹⁴⁾

4. LIPOPROTEIN-ASSOCIATED PROTEINS

4.1. ApoB Human apoB-100, a 4536-amino acid-secretory glycoprotein, has a particularly critical role in the assembly of VLDL in the liver.¹⁴⁾ A unique mRNA editing in the small intestine generates a truncated form of apoB corresponding to its N-terminal 48% (apoB-48), which directs the formation of CM.⁹⁵⁾ ApoB-100 plays a particular role in maintaining the structural integrity of lipoprotein particles and in controlling their interaction with LDLR.⁹⁶⁾ An LDL particle contains a single apoB-100 molecule together with about 3000 lipid molecules. Sixteen *N*-glycosylation sites in apoB-100 are glycosylated with high-mannose and complex forms of oligosaccharides.^{97,98)} ApoB is highly insoluble in aqueous solutions and remains at the lipoprotein particle throughout its metabolism. From the circular dichroism (CD) spectra of LDL, the secondary structure of apoB-100 is characterized by a large content of α -helix (*ca.* 50%) and smaller amounts of β -sheet (*ca.* 11%), β -turn (*ca.* 27%) and random coil (*ca.* 13%).⁹⁹⁾ The lipid-binding property of apoB is ascribed to a series of amphipathic α -helical and β -strand domains arranged in the following order: NH₂- $\beta\alpha_1$ - β_1 - α_2 - β_2 - α_3 -COOH.²⁾ The $\beta\alpha_1$ domain represents a globular region.²⁾ The amphipathic α -helices of the α_2 and α_3 domains are similar to those found in exchangeable apolipoproteins.²⁾ The amphipathic β -strands are the anchors keeping apoB bound to the lipoprotein surface.^{100,101)} The portion of apoB-100 that forms amphipathic β -sheets interacts directly with the lipid core.¹⁴⁾ The irreversibly lipid-associating β -sheet regions of apoB-100 are key structural areas for the integrity of LDL particles.

The first 89% of apoB-100 enwraps the LDL particle like a thick ribbon, completing the encirclement by approximately amino acid residue 4050, the junction of the β_2 and the α_3 domains.¹⁰²⁾ There is a kink in the ribbon beginning almost halfway along its length at the start of the α_2 domain.¹⁰²⁾ The C-terminal 11% of apoB-100, α_3 domain, constitutes the bow-like structure of about 480 residues beginning at residue 4050, which stretches back and then crosses over the ribbon between residues 3000 and 3500.¹⁰²⁾

ApoB-containing lipoprotein assembly requires the PL transfer activity of MTP.¹⁰³⁾ In the absence of MTP, apoB folds incorrectly and undergoes proteasomal degradation. In the setting of apoB deficiency, although the smooth ER contains lipid droplets, no droplets are observed in the Golgi and the intercellular space.¹³⁾ When apoB synthesis occurs, lipoprotein particles exist in the Golgi and the intercellular space. ApoB is required for the transport of lipoproteins from the ER to the Golgi, and its structure may play an important role in the lipoprotein secretion.

ApoB-100 is responsible for the binding to LDLR but not other members of the LDLR family.^{2,104)} In the genetic disorder familial defective apoB-100, high levels of LDL accumulate in the circulation, which leads to hypercholesterolemia and premature atherosclerosis.¹⁰⁵⁾ Familial defective apoB-100 is caused by a mutation of apoB-100 (R3500Q), which disrupts the binding of LDL to LDLR.¹⁰⁵⁾ Another mutation R3531C is

also associated with a decrease in LDLR binding.¹⁰⁶⁾ LDLR binding sites exist in the α_3 domain of apoB-100.¹⁰⁷⁾ Three long positively charged amphipathic α -helices, residues 3147–3157, 3170–3208, and 3359–3367, are termed site A, site C, and site B, respectively.²⁾ Site B is the domain interacting with LDLR, whereas site A is not the primary LDLR binding site.^{2,108)} Arg3500 is not directly involved in receptor binding, although the R3500Q mutation of apoB-100 leads to inability of the LDL particles to interact with LDLR.¹⁰⁸⁾ However, the removal of 20% C-terminal domain in the R3500Q mutant results in the normal receptor-binding activity, indicating that the carboxyl-terminal 20% of apoB-100 is necessary for the R3500Q mutation to disrupt receptor binding.¹⁰⁸⁾ The R3500Q mutation has been proposed to induce a conformational change in the C-terminal tail.⁵⁶⁾

ApoB-48 is capable of binding PGs despite lacking site B.¹⁰⁹⁾ In addition to LDLR binding, apoB-100 contains at least eight potential PG-binding sites.⁶⁰⁾ The binding of LDL to PGs is mediated by ionic interactions between the negatively charged sulfate and carboxyl groups of the GAGs and the positively charged lysine and arginine residues in apoB-100.⁵⁴⁾ The binding of LDL particles to GAGs is severely impaired by the mutation of Lys3363 of apoB-100.⁵⁶⁾ Based on the number of available binding sites, the affinity of apoB-48 for PGs is greater than that of apoB-100, suggesting that apoB-100 masks the high-affinity sites present on apoB-48.^{47,110)}

4.2. ApoE ApoE, a 299-amino acid plasma apolipoprotein, is a ligand for LDLR and LRP, and binds to cell surface HSPGs.¹¹¹⁾ ApoE has seven amphipathic helical segments that are responsible for lipid binding.¹¹²⁾ Based on the binding model of apolipoproteins, the apolipoprotein helices are predicted to deeply insert into the surface layers, such that the amphipathic helices are buried within the hydrophobic interior of PL monolayers.¹¹³⁾ Apo E is tetrameric in aqueous solution but monomeric on the lipid particle surfaces.¹¹⁴⁾ ApoE contains two independently folded domains, the 22-kDa N-terminal domain and 10-kDa C-terminal domain.¹¹¹⁾ The C-terminal domain has a high affinity for lipid and is responsible for lipoprotein binding and preference.¹¹⁵⁾ The N-terminal domain exists in the lipid-free state as a four-helix bundle and contains the LDL receptor-binding region.¹¹⁶⁾ The cluster of arginine and lysine residues located between residues 136–158 represents the binding site for LDLR. The segment comprising the receptor-binding portion of apoE (residues 130–149) interacts with ligand-binding clusters of LRP.^{117,118)} Both domains of apoE contain heparin-binding sites, but the N-terminal site plays a dominant role in the binding to heparin.¹¹⁹⁾ The cluster of positively charged amino acids between residues 136–150 is also involved in the heparin interaction. The four-helix bundle in the N-terminal domain undergoes a conformational opening upon lipid binding, leading to the receptor-active conformation of apoE.^{111,120)} In this conformation, the positive electrostatic potential in the receptor-binding region of apoE is enhanced.^{121,122)} The leucine zipper motif confers stability to the helix bundle conformation of the N-terminal domain, which serves to maintain apoE in a receptor-inactive state.¹²³⁾ It has been proposed that apoCs and/or lipid composition induce conformational alteration of the N-terminal domain of apoE and modulate the receptor binding properties, between an open lipid-bound receptor-active state and a globular receptor-inactive state.¹²⁴⁾

The liver produces the vast majority of plasma apoE, and apoE promotes the internalization of TG-rich lipoprotein remnants by hepatocytes. CM contains essentially no apoE, whereas their remnants acquire apoE from other lipoprotein classes.¹¹²⁾ ApoE is lost from the surface of LDL particle. Patients lacking apoE accumulate remnant lipoproteins.¹²⁵⁾ ApoE knockout mice have high levels of plasma cholesterol and readily develop atherosclerosis.^{126,127)} ApoE knockout mice contain large amounts of circulating apoB-48-containing lipoproteins that cannot interact with LDLR and LRP.⁷⁾ Catabolism of these lipoproteins is highly dependent on the HSPG-mediated removal pathways.

Within atherosclerotic lesions, lipoproteins contain apoE in addition to apoB-100.¹²⁸⁾ Lipoprotein remnants with apoE are avidly taken up by and markedly stimulate the CE accumulation in macrophages.¹²⁹⁾ In the lesions, most of the apoE molecules are synthesized locally by resident macrophages.¹³⁰⁾ Additionally, apoE directly modifies macrophage-mediated immune responses that contribute to atherosclerosis.⁶⁸⁾

The genetic disease type III hyperlipoproteinemia is characterized by impaired clearance of lipoprotein remnants, as a result of various mutant forms of apoE that are defective in binding to lipoprotein receptors.¹⁾ ApoE exists as one of three predominant isoforms.^{112,124)} ApoE3 isoform is the most abundant isoform and possesses a single cysteine residue at position 112 with arginine at position 158. ApoE4 isoform contains an arginine at position 112, while apoE2 isoform has cysteines at both positions 112 and 158. Isoforms apoE3 and apoE4 bind to hepatic lipoprotein receptors with high affinity, whereas apoE2 binds only weakly.¹³¹⁾ The LDLR-binding activity of apoE2 isoform is dramatically reduced (less than 2% of normal apoE3 activity), which is associated with type III hyperlipoproteinemia.¹³²⁾ The altered salt bridge interaction in apoE2 induces the deformation of the receptor-binding region.¹³³⁾ Nevertheless, the majority of apoE2 homozygotes do not exhibit hyperlipidemia or significant accumulation of remnant lipoproteins.¹⁾ ApoE2 has significant HSPG-binding activity (50–90% of normal apoE3 activity).¹³⁴⁾ ApoE also plays essential roles in the transport of lipids in the nervous system.¹⁾ ApoE4 isoform is known to be a risk factor for Alzheimer's disease.^{135,136)}

ApoCs inhibit the apoE-dependent recognition of TG-rich lipoproteins by LDLR and LRP.^{137,138)} The apoE-mediated hepatic uptake of plasma lipoproteins is controlled by the ratio of apoE to apoCs rather than the absolute apoE content.⁴⁶⁾

At high concentrations, apoE leads to hypertriglyceridemia.^{139,140)} The apoE content is inversely correlated with the LPL-mediated lipolysis rate of VLDL *in vitro*.¹⁴¹⁾ ApoE efficiently inhibited the LPL-mediated lipolysis of TG-rich emulsions *in vivo* and *in vitro*.¹⁴²⁾

4.3. ApoC-I ApoC-I is primarily expressed in the liver and consists of 57 amino acid residues.⁴⁶⁾ ApoC-I residues 7–24 and 35–53 are important for the binding to lipoproteins and adopt well-defined amphipathic helices with distinct hydrophobic and hydrophilic faces.¹⁴³⁾ ApoC-I has inhibitory effects on the LPL activity and on the lipoprotein binding to LDLR and LRP.^{46,144)} Among apoCs, apoC-I is the most potent inhibitor for the apoE-mediated binding of lipoprotein remnants to LRP.¹⁴⁵⁾ ApoC-I displaces apoE from TG-rich lipoproteins, and directly interferes with their hepatic clearance.⁴⁶⁾ In addition to the displacement of apoE from the particles,

apoC-I binding may induce a change in the conformation of apoE bound on the particles and abolish the ability of apoE to interact with LRP. ApoC-I activates the cholesterol esterification by LCAT to a lesser extent than apoA-I, the most powerful activator.¹⁴⁶⁾ On the other hand, apoC-I is an inhibitor of CETP.^{147,148)}

Transgenic mice overexpressing human apoC-I exhibit elevated levels of cholesterol and TG owing to the accumulation of cholesterol-enriched VLDL, VLDL remnants, and LDL.^{149,150)} In human apoC-I-transgenic mice, the elevated lipid levels are primarily associated with decreased uptake of apoB-containing lipoproteins by the liver rather than with disturbed lipolysis of lipoproteins.^{149,150)} Surprisingly, *ApoC1*-knockout mice show normal serum lipid levels on a chow diet.¹⁵¹⁾

4.4. ApoC-II ApoC-II, a 79-amino acid protein, is mainly expressed in the liver and intestine and is an essential activator of LPL. ApoC-II contains three α -helices spanning approximately residues 16–38, 45–57, and 65–74.¹⁵²⁾ The structures of apoC-II involved in lipid interactions reside at the N-terminus (residues 1–51).⁴⁶⁾ The helical structure close to the C-terminal end of apoC-II is important for the activation of LPL.¹⁵³⁾ ApoC-II may bind directly to LPL and activate LPL after binding of LPL to the surface of TG-rich lipoproteins.⁴⁶⁾ The lipid-binding domain of apoC-II is required for the stimulation of LPL activity.¹⁵⁴⁾ At high protein levels, however, apoC-II inhibits LPL activity.⁴⁶⁾ ApoC-II also has an inhibitory effect on the HL-mediated lipolysis to a lesser extent than apoC-III.¹⁵⁵⁾ Both apoC-II and apoC-III inhibit LCAT activity, presumably due to the displacement of the activating apolipoproteins from the lipoprotein surface.¹⁵⁶⁾

Genetic deficiency of apoC-II causes severe hyperchylomicronemia.^{27,46)} Patients with genetic defects in apoC-II display high circulating levels of TG.^{157,158)} Remarkably, transgenic mice overexpressing human apoC-II show accumulation of TG-rich VLDL particles in the circulation.¹⁵⁹⁾ The high levels of apoC-II inhibit the lipolysis of VLDL and/or the uptake of the VLDL particles by the liver.

4.5. ApoC-III ApoC-III is a 79-amino acid glycoprotein synthesized mainly in the liver and to a minor extent in the intestine as a component of chylomicrons, VLDL, LDL and HDL.¹¹¹⁾ In normotriglyceridemic subjects, apoC-III is associated primarily with HDL, whereas there is redistribution of apoC-III to chylomicrons and VLDL in the postprandial state and hypertriglyceridemia.^{160,161)} ApoC-III consists of two helical domains. The N-terminal domain of apoC-III is important in the modulation of LPL activity, and the binding of apoC-III to surface PL is mediated by the C-terminal helix.^{46,162)} In the three-dimensional NMR structure of apoC-III in complex with sodium dodecyl sulfate (SDS) micelles, 6–10-residue amphipathic helices wrap around the micelle surface, three positively charged residues line the polar faces of helices 1 and 2, and an array of negatively charged residues lines the polar faces of helices 4 and 5 and the adjacent flexible loop.¹⁶³⁾ ApoC-III molecules are separated into three isoforms that differ in their degree of O-linked sialylation at the threonine residue in position 74.⁴⁶⁾ The intracellular glycosylation of apoC-III is not necessary for its secretion from cells and ability to bind plasma lipoproteins.¹⁶⁴⁾

ApoC-III plays a role as a potential inhibitor of the LPL-mediated lipolysis, both by a direct interaction with LPL and

by interfering with lipoprotein binding to the cell surface GAG matrix where lipolytic enzymes reside.¹⁴⁴⁾ The mechanism of apoC-III-LPL interaction is accounted for by the lipid-binding part of molecules or attributed to protein-protein interactions that are independent of lipid binding.^{162,165)} ApoC-III also inhibits HL.¹⁵⁵⁾

ApoC-III completely abolishes the apoB-100-mediated binding of lipoproteins to LDLR, suggesting that this inhibitory action of apoC-III on lipoprotein binding is due to a masking of the receptor domain of apoB-100 by apoC-III.¹⁶⁶⁾ Increased levels of apoC-III on the lipoprotein particles displace apoE, which may result in decreased remnant clearance.¹⁶⁷⁾

The human apoC-III gene is located in a gene cluster together with the apoA-I and apoA-IV genes on the long arm of chromosome 11.¹⁶⁸⁾ ApoC-III deficiency is usually associated with apoA-I and apoA-IV deficiency, making it difficult to estimate the respective contributions of these deficiencies.^{169,170)} Patients with hereditary deficiency of apoC-III and apoA-I had low plasma TG and accelerated clearance of both CM and VLDL.^{171,172)} Homozygosity of the A641C allele in the apoC-III gene promoter is associated with significantly lower serum levels of apoC-III,¹⁷³⁾ whereas the variant alleles in the apoC-III gene promoter (C482T and T455C) cause a 30% increase in the fasting plasma apoC-III concentration compared with the wild-type homozygotes.¹⁷⁴⁾

ApoC-III in VLDL and LDL is an independent risk factor for coronary heart disease.¹⁷⁵⁻¹⁷⁷⁾ A high concentration of apoC-III-containing VLDL is associated with the delayed catabolism of TG in VLDL and hypertriglyceridemia.^{178,179)} ApoC-III enhances the conversion from buoyant LDL to dense LDL.^{180,181)} ApoC-III deficiency (by *ca.* 50% of normal levels) confers a favorable lipid profile and reduced subclinical coronary artery atherosclerosis.¹⁸²⁾ The polymorphisms in the apoC-III gene promoter are associated with plasma apoC-III concentration, plasma TG concentration, lipoprotein profile, cardiovascular health, nonalcoholic fatty liver disease, insulin sensitivity, and longevity.^{173,174)} Human apoC-III transgenic mice exhibit markedly elevated levels of CM and VLDL TG.¹⁸³⁾ In addition, a synergistic interaction between the human apoC-III transgene and LDLR defects produces large quantities of VLDL and LDL and enhances the development of atherosclerotic lesions in mice.¹⁸⁴⁾ ApoC-III delays the metabolism of TG-rich lipoproteins by inhibiting LPL activity and apoE-dependent hepatic uptake, which may increase the probability of cholesterol deposition of lipoprotein particles in the vessel wall.^{177,178)}

4.6. ApoA-I ApoA-I, a 243-amino acid protein, is the major apolipoprotein component of HDL, serving as an activator of LCAT and functioning as an acceptor of cell membrane FC in the reverse cholesterol transport pathway.^{111,185)} ApoA-I mediates the formation of nascent HDL particles from cellular FC and PL through the interaction with the cell surface transporter ABCA1.^{186,187)} The majority of apoA-I is secreted from the liver. ApoA-I molecules are also accommodated in CM. ApoA-I is comprised of an N-terminal domain (residues 1-43) and the remaining region containing eight 22- and two 11-amino acid amphipathic α -helices.¹¹¹⁾ ApoA-I may initially bind to a lipid surface through amphipathic α -helices in the C-terminal domain, followed by opening of the helix bundle in the N-terminal domain.¹⁸⁸⁾

4.7. LPL LPL catalyzes the hydrolysis of plasma TG,

diglycerides, PC, and phosphatidylethanolamine transported in CM and VLDL.¹⁸⁹⁾ The hydrolyzing activity of LPL toward PL is much lower than that toward TG.¹⁹⁰⁾ LPL is synthesized primarily in adipose and skeletal muscle. LPL is transported to the endothelial surface and is bound to HSPGs.³¹⁾ LPL binding to endothelial cells is not a static situation. Some LPL dissociates from the cells, and some LPL is internalized and recycled to the cell surface.¹⁹¹⁾ LPL is also expressed in the nervous system, adrenals, macrophages, proximal tubules of the kidneys, pancreatic islet cells, and lungs.¹⁹²⁾ The LPL activity in pancreatic islet cells is related to insulin secretion and lipotoxicity.¹⁹³⁾

The cDNA of LPL codes for a 475-amino-acid protein including a 27-amino-acid signal peptide. The catalytic center is formed by three amino acids: Ser132, Asp156 and His241.¹⁹²⁾ The enzymatic activity of LPL is localized within its N-terminal domain, and the C-terminal domain of LPL contains binding sites for lipoproteins and for specific cell-surface receptors, including HSPGs and members of the LDLR family.¹⁹⁴⁻¹⁹⁷⁾ LPL can enhance the binding and uptake of several classes of lipoproteins through bridging between the lipoproteins and HSPGs.^{194,195)} Furthermore, LPL enhances the uptake of remnants partially mediated by the ability of LPL to directly bind to LRP and LDLR.^{196,197)} The hydrolytic activity of LPL is not required for this bridging function.^{195,197)}

For maximal rates of catalysis, LPL requires apoC-II.¹⁸⁹⁾ ApoC-II does not increase the binding of LPL to a lipid surface.¹⁸⁹⁾ LPL is capable of hydrolyzing TG present in the PL monolayer.¹⁹⁸⁾ LPL may interact with the head group region of PL rather than with the hydrophobic interior of the surface monolayers.¹⁹⁹⁾ LPL binds to the lipid surface without marked changes in the surface structure, suggesting superficial binding of LPL to the PL surface.²⁰⁰⁾

In the rare autosomal recessive disorder of familial LPL deficiency (type I hyperlipoproteinemia), the near absence of LPL activity results in fasting hypertriglyceridemia.¹⁸⁹⁾ Heterozygotes for LPL deficiency have abnormalities in postprandial lipemia despite relatively normal fasting TG.²⁷⁾ There are several polymorphisms of LPL that are associated with hypertriglyceridemia.^{192,201)}

The LPL activity is found in greater amounts in the atherosclerotic lesions than in the normal arteries.²⁷⁾ In the medium and large arteries, LPL is present on the luminal endothelial surface and in the macrophage-rich areas within the plaque. LPL is expressed by macrophages within the vessel wall. Increased LPL activity in the arterial wall is correlated with increased areas of lipid deposition and increased atherosclerotic lesion formation.⁸⁾ LPL activity in mouse macrophages is correlated with their propensity to develop atherosclerosis.²⁰²⁾ Macrophage-derived LPL shows a proatherogenic role in LDLR-deficient mice.²⁰³⁾ LPL forms macromolecular aggregates by simultaneously binding to both matrix PGs and apoB-containing lipoproteins, which increases their retention by subendothelial matrix and uptake by cells.²⁰⁴⁾

5. LIPOPROTEIN RECEPTORS

5.1. LDLR LDLR, a 160 kDa protein, plays a major role in plasma LDL uptake and is expressed in all mammalian cell types.²⁰⁵⁾ LDLR binds lipoproteins containing apoB-100 and/or apoE.¹³¹⁾ LDLR contains seven imperfect, ligand-binding

repeats located at the N-terminus.²⁰⁶⁾ Each repeat is *ca.* 40 amino acids in length, binds calcium and is characterized by an abundance of cysteines and negatively charged aspartate and glutamate residues.

Familial hypercholesterolemia is a genetic defect of LDLR. In patients with familial hypercholesterolemia, poor removal of LDL particles from the circulation and LDL accumulation results in an increase in plasma cholesterol concentrations. Nevertheless, lipoprotein remnants are cleared rather normally in the patients.¹⁾

Proprotein convertase subtilisin/kexin type 9 (PCSK9) promotes the degradation of hepatic LDLR and reduces the efficiency of endocytic function.^{49,207)} Secreted PCSK9 decreases the number of LDLR in mice hepatocytes. Sequence variations in PCSK9 lead to decreased plasma LDL and result in a substantial reduction in the incidence of atherosclerotic heart disease.²⁰⁸⁾ In heterozygous familial hypercholesterolemia, two monoclonal antibodies against PCSK9, alirocumab and evolocumab, diminish plasma LDL cholesterol, apoB and Lp(a).^{209,210)} Upregulation of LDLR may also increase the plasma clearance of Lp(a).

5.2. LRP LRP is a 600 kDa multiligand receptor, which is expressed strongly in hepatocytes and binds apoE-containing remnant lipoproteins and other proteins.²¹¹⁾ LRP has multiple ligands, including apoE, HL, LPL, and activated α_2 -macroglobulin.¹⁾ LRP recognizes apoE but not apoB-100. The ability of LRP to bind numerous structurally distinct ligands with high affinity arises from the ligand-binding sites with unique contour surface and charge distribution.²¹¹⁾ LRP is structurally similar to other members of the LDLR gene family. LRP consists of five common structural units, ligand-binding type cysteine-rich repeats, epidermal growth factor receptor-like cysteine-rich repeats, YWTD domains, a single membrane-spanning segment, and a cytoplasmic tail.²¹¹⁾

The receptor-associated protein, a 39 kDa protein, exists in the ER and is a specific inhibitor of ligand binding to LRP.²¹¹⁾ In receptor-associated protein knockout mice, the total amount of LRP present in the liver is reduced by 80%²¹²⁾ which is caused by the aggregation of LRP in the ER and subsequent intracellular degradation.²¹³⁾ LRP also serves as the receptor for α_2 -macroglobulin. The activated α_2 -macroglobulin can compete with lipoprotein remnants for LRP binding.¹⁾

LRP is also present in macrophages and vascular smooth muscle cells from atherosclerotic lesions and normal vessels.^{214,215)} LRP, contrary to LDLR, is not regulated by intracellular cholesterol.

5.3. HSPG HSPGs are members of the family of PGs, which are components of cell membranes and extracellular matrix.^{1,27,55)} The two major parts of the PG molecule are GAGs and core proteins. HSPGs are polyanionic macromolecules that contain a core protein with multiple HS chains covalently attached. In the liver, the major core proteins are syndecans, although glypican, betaglycan and perlecan are also present.²¹⁶⁾ HS is a polymer composed of repeating disaccharide units of a hexuronic acid (either glucuronic acid or iduronic acid) and glucosamine. The glucosamine residues are either *N*-acetylated or *N*-sulfated, and both hexuronate and glucosamine residues are *O*-sulfated in varying positions.²¹⁷⁾ Heparin differs from HS in the extent of *N*-acetylation, *N*-sulfation and *O*-sulfation, and in the content of iduronate.²⁷⁾ Hepatic HSPGs are especially diverse, highly sulfated, and

rich in heparin-like domains.¹⁾

The major HSPG in the subendothelial matrix is perlecan.²¹⁸⁾ The contents of CSPGs and DSPGs are increased in atherosclerosis. In contrast, HSPGs negatively correlate with human atherosclerosis, aging and diabetes.^{219,220)} The amount of cholesterol accumulated in the lesion is inversely correlated to the amount of HS.

HSPGs act as potential receptors for lipoprotein remnants or facilitate the uptake by ligand transfer to LRP. The HSPG-LRP pathway mediates the internalization either by transfer of the remnants from HSPGs to LRP or by binding of the lipoprotein remnants to HSPGs complexed with LRP.¹⁾ HSPGs alone can function as a receptor and mediate the uptake of lipoprotein remnants.²²¹⁾

Heparinase releases the sulfated GAG side chains from HSPGs and inhibits the plasma clearance and liver uptake of remnant lipoproteins.²²²⁾ The treatment of a variety of cells with heparinase significantly inhibited the binding and uptake of apoE-enriched remnant lipoproteins.²²³⁾ Heparin impedes the binding of apoE-containing lipoproteins with HSPGs.²²¹⁾ Lactoferrin, an iron-binding glycoprotein, impairs the clearances of CM and VLDL and decrease the liver uptake by inhibiting and blocking interaction of apoE with LRP and HSPGs.^{224,225)} This inhibitory effect of lactoferrin is attributed to an arginine/lysine-rich sequence at position 25-31 that resembles the receptor-binding region of apoE.²²⁶⁾

6. LIPID COMPONENTS

6.1. SM SM is one of the major lipids in lipoproteins. The liver synthesizes sphingolipids *de novo* and incorporates the newly synthesized SM into VLDL.²²⁷⁾ The ratio of SM/PC widely varies among lipoprotein subclasses and lipoprotein SM concentration is affected by several factors.²²⁸⁻²³⁴⁾ The SM/PC ratio in VLDL is 0.25. Unlike PC, SM is not degraded by LPL, HL, or LCAT, and the transfer of SM among lipoproteins as well as between lipoproteins and cell membranes is slower than that of other PL.²³⁵⁻²³⁷⁾ SM removal from plasma is absolutely dependent on hepatic clearance. Thus, SM becomes enriched in VLDL remnants, and the SM/PC ratio in LDL is quite high (0.5).²³⁸⁾ In hypercholesterolemic patients, the SM/PC molar ratio in LDL was increased to 0.522.²²⁹⁾ Lipoprotein SM concentration is raised by lipopolysaccharide, dietary cholesterol, casein, and olive oil, and the SM/PC ratio was higher in all lipoproteins in *apoE* knockout mice compared with wild-type mice.^{230,231,233,234,239)} Plasma SM levels show a significant, although moderate, correlation with remnant cholesterol levels; however, in multivariate analysis, plasma SM level remains as a significant predictor of CHD even after additional adjustment for remnant lipoprotein cholesterol levels.²⁴⁰⁾ LDL extracted from human atherosclerotic lesions is highly enriched in SM compared with plasma LDL.²³⁸⁾

The hydrophobic region of natural SM consists of highly saturated acyl chains and thus SM has more ordered acyl chain structures than PC.¹⁹⁹⁾ Both the amino and hydroxyl groups of sphingosine participate in intramolecular and intermolecular hydrogen bonding, which are not found in PC. The hydrogen bonding capacity of SM in the backbone region, in addition to a condensed acyl chain organization, results in increased packing and hence decreased hydration of the head group region.¹⁹⁹⁾ I have revealed that the incorporation of SM

into lipid emulsions markedly increases acyl chain order and decreases head group hydration of the surface monolayers¹⁹⁹⁾ (Fig. 1B). Therefore, SM is a structural and functional determinant at the lipoprotein surface.

The presence of SM delays the removal of emulsion particles from animal plasma.^{241–243)} SM strongly inhibits the LPL-mediated lipolysis in the emulsion particles.^{198,199,243)} Kinetic studies of the lipolysis rates have shown that the incorporation of SM into the emulsion surface causes an increase in K_m^{app} and a decrease in V_{max}^{app} , indicating that SM inhibits the lipolysis by decreasing both affinity for substrates and catalytic activity of LPL.^{198,199)} The lipid binding of LPL is presumed to depend on the structure of the head group region, which is affected by the presence of SM.¹⁹⁹⁾ It has been suggested that the strong condensation in the head group region caused by SM inhibits LPL binding to the lipid surface and thus decreases the LPL activity.¹⁹⁹⁾

To clarify the role of SM in lipoprotein uptake, I have prepared lipid emulsions containing triolein (TO), PC and SM as model particles of lipoproteins. ApoE binding studies have revealed that the incorporation of SM into the emulsion surface reduces the binding capacity of apoE without changing the affinity.²⁴⁴⁾ The increased surface packing in SM-containing emulsions may therefore result in the decrease in the insertion of apoE into the surface layer. SM in the emulsion surfaces reduces the apoE-mediated uptake of the particles by HepG2 human hepatoma cells, which is consistent with the decreased binding amount of apoE to the SM-containing emulsion surfaces.²⁴⁴⁾ The receptor-active state of apoE may be unfavorable for the SM-containing particles because of the increased packing stress. The addition of lactoferrin almost abolishes the effect of apoE on the emulsion uptake, indicating that lactoferrin-sensitive pathways contribute to the apoE-mediated uptake of the emulsion particles.²⁴⁴⁾ The treatment of the cells with heparinase results in decreased uptake of the emulsion particles in the presence of apoE.²⁴⁴⁾ Therefore, HSPGs and LRP are mainly involved in the apoE-mediated uptake of lipid emulsions by HepG2 cells.^{244,245)} The apoE-mediated uptake of the lipid emulsions is inhibited by the presence of apoC-II or apoC-III.^{244,245)} SM in the emulsion surface enhances the inhibitory effects of apoC-II and apoC-III on the apoE-mediated uptake of emulsion particles.²⁴⁴⁾ The incorporation of SM into the emulsion surface reduces the amount of apoE binding in human plasma and increases the ratios of apoC-II and apoC-III to apoE.²⁴³⁾ More displacement of apoE by apoC-II or apoC-III from SM-containing particles may result in greater reduction of cell uptake.

Strikingly, LPL promotes the uptake of emulsion particles into HepG2 cells.²⁴⁴⁾ However, the effect of LPL on the emulsion uptake is decreased by replacing surface PC with SM.²⁴⁴⁾ It is conceivable that the mechanism for the inhibition of LPL-mediated uptake is the impaired binding of LPL to the SM-containing particles. In the presence of LPL, lactoferrin almost prevents the emulsion uptake, indicating that HSPGs and LRP are the major contributors to the LPL-mediated uptake of lipid emulsions by HepG2 cells.²⁴⁴⁾ Taken together, these findings suggest that the SM-induced changes in the binding properties of apolipoproteins and LPL are associated with decreased hepatic uptake of lipoproteins.

6.2. FC The amount of FC in lipoproteins regulates their metabolism. FC is a major surface lipid of CM remnants.

When CM is converted to chylomicron remnant by the action of LPL, the relative content of FC increases in the remnant.²⁴⁶⁾ The FC/PL ratio of the remnants in human serum in the postprandial state is close to 1.²²⁸⁾ The FC/PL ratios in VLDL and LDL are 0.78 and 0.73, respectively.²⁴⁷⁾

FC can distribute between the surface and core phases in lipid emulsions and lipoproteins. From the surface-core phase equilibrium, over 80% of FC is accommodated in the surface phase in emulsions consisting of TG and PC.²⁴⁸⁾ FC induces a motional restriction of PC and a rigidification of the emulsion surface with increasing content.²⁴⁸⁾ FC is associated with surface PC but located deep in the inner hydrocarbon region.²⁴⁸⁾ I have demonstrated that FC increases the acyl chain order and head group hydration of the surface PC layer of emulsions²⁴⁹⁾ (Fig. 1B). The acyl chain region of FC-containing emulsion surface layer is more rigid than that of emulsion surface without FC. Moreover, FC enrichment on the emulsion surface leads to the separation and hydration of PC head groups.

The amount of FC in emulsion particles regulates the metabolism by affecting the binding of apolipoproteins to the particle surface. Emulsions with a high content of FC bind less apoA-I, apoA-IV and apoCs, and the relative amount of apoE is increased.²⁵⁰⁾ The presence of FC facilitated the removal of emulsion particles from plasma.²⁴³⁾ Interestingly, FC does not affect the lipolysis rates although both TG solubility in PC monolayer and apoC-II binding are decreased, indicating that neither TG solubility nor amount of apoC-II binding are determinants of LPL-mediated lipolysis.¹⁹⁸⁾

I have used lipid emulsions containing TO, PC and FC as a model for TG-rich lipoprotein remnants.^{245,249,251)} Emulsions rich in FC are metabolized like CM remnants.²⁵⁰⁾ FC markedly increases the apoE-binding maximum of emulsions without changing the binding affinity.²⁴⁹⁾ The binding maximum of apoE is correlated with the hydration between PL polar head groups at the emulsion surface, indicating that the PL head group separation plays a crucial role in apoE binding.²⁴⁹⁾ At the emulsion surface, the inverse wedge-shaped lipid, FC, releases the stress and increases the number of binding sites for apoE. Although both SM and FC at the emulsion surface increase the acyl chain packing, the apoE-binding maximum is lowered by SM but elevated by FC.²⁴⁹⁾ Thus, an enhancement in the apoE-binding maximum of FC-containing emulsions may be caused by an increase in PC head group separation but not by an increase in acyl chain packing.

FC in the emulsion surface increases the amount of apoE bound to the particles, but had no effect on the binding amount of apoC-III, suggesting that the binding of apoC-III is insensitive to the degree of PC head group separation at the emulsion surface.²⁴⁵⁾ ApoC-III reduces the binding of apoE to the emulsion particles.²⁴⁵⁾ The binding amount of apoE correlates inversely with the binding amount of apoC-III to the lipid emulsions.²⁴⁵⁾ Surface FC alleviates the inhibitory effect of apoC-III on apoE binding to the emulsion surface, and increases the total binding of apoE and apoC-III.²⁴⁵⁾ FC at the emulsion surface may increase the number of the apoE binding sites rather than the apoC-III binding sites.

FC in the emulsion surface enhances the apoE-mediated uptake by J774 mouse macrophages by increasing the amount of apoE bound to the emulsion particles.²⁴⁹⁾ Lactoferrin or heparin treatment dramatically prevents the apoE-mediated uptake of the emulsions into J774 macrophages, suggesting

that HSPGs and LRP are predominantly involved in the apoE-mediated uptake.²⁴⁹⁾ In the presence of apoE, the uptake of FC-containing emulsions by HepG2 cells is higher than that of emulsions without FC.²⁴⁵⁾ The apoE-mediated uptake of the emulsion particles decreases with apoC-III concentration.²⁴⁵⁾ Despite a sufficient amount of apoE bound to FC-containing emulsions, apoC-III almost abolished the cellular uptake of the emulsions *via* HSPG and LRP pathways.²⁴⁵⁾ Excluding the case of FC-containing emulsions in the presence of both apoE and apoC-III, the emulsion uptake is positively correlated with the amount of apoE bound to emulsions.²⁴⁵⁾ In the presence of apoE, there are inverse correlations between the emulsion uptake and the binding of apoC-III.²⁴⁵⁾ Thus, apoC-III bound to emulsion particles may prevent the apoE-mediated uptake. It is possible that at the FC-containing surface, apoC-III leads to the receptor-inactive conformation of the apoE-N-terminal domain or masks the cluster of positively charged amino acids of apoE involved in the binding to LRP and HSPG, in addition to the attenuation of apoE binding to the particle surface. These observations suggest that apoC-III impedes the binding and activation of apoE at the lipoprotein surface. Furthermore, FC at the surface of lipoprotein remnants may modulate these functions of apoC-III.

6.3. CE CE, along with TG, is the major core component of plasma lipoproteins. During the transport of plasma TG-rich lipoproteins, the CE content of these lipoproteins is progressively increased by the action of CETP. The increase of CE replaces the loss of TG in the hydrophobic core of these particles. The core replacement of TG with cholesteryl oleate (CO) has little effect on the surface rigidity, despite the large difference in the core mobility.²⁴⁸⁾ However, in the presence of FC, the core replacement with CO results in a marked increase in the surface rigidity.²⁴⁸⁾ Although FC preferentially partitions into the surface, the incorporation of CO into core TO increases the distribution of FC into the core phase, arising from the association of FC with CO in the core. Over 80% of the FC is associated with the surface phase in emulsions consisting of TG and PC, whereas only about one-half of the FC is located at the surface in emulsions consisting of CO and PC.²⁴⁸⁾ The core replacement with CO modulates the surface properties of the emulsion particles through the redistribution of FC in the surface layers.

The increased amount of CO in the emulsion core retards the emulsion clearance from rat plasma.²⁵²⁾ Replacing core TO with CO markedly decreases the apoE binding capacity to emulsion particles without changing the binding affinity.²⁵²⁾ The uptake of TO-PC emulsions into HepG2 cells is greatly increased by the addition of apoE, but the effect of apoE decreased with increasing CO content.²⁵²⁾ The physical state of core lipids is modulated by the content of CE and plays a crucial role in lipoprotein metabolism.

6.4. Particle Size Apo-B100 binds to LDLR only after the conversion of large VLDL to smaller LDL.¹⁰⁴⁾ The removal of the C-terminus of apo-B100 on VLDL dramatically enhances the apoB-mediated receptor-binding activity.¹⁰⁸⁾ It has been proposed that, during the lipolysis of VLDL to LDL, the C-terminus of apoB-100 changes its conformation to allow interaction with LDLR.¹⁰⁸⁾ The conformational changes in apoB-100 are limited to particular regions in the metabolic cascade from VLDL to LDL.²⁵³⁾

ApoE displays no binding preference between small and

large emulsion particles.²⁵⁴⁾ However, apoE enhances the uptake of both VLDL-size and remnant-size emulsion particles by J774 macrophages, but the effect is greater on the uptake of larger particles compared to the uptake of smaller particles.²⁵⁵⁾ Addition of apoC-III almost completely displaces apoE from small VLDL particles, while larger VLDL contain tightly bound apoE, which are not displaced by apoC-III.²⁵⁶⁾ The ratio of apoCs/apoE on the emulsion particles in serum is inversely correlated with particle size, and the particle size shows a positive correlation with the association of the emulsions with the liver.²⁵⁷⁾

ApoCs are mainly associated with HDL in the fasting state, whereas in the fed state, they preferentially redistribute to the surfaces of CM and VLDL particles.⁴⁶⁾ CM-size lipid particles do not take up free apoA-I and apoA-IV but rather apoCs in the presence of HDL.²⁵⁸⁾ ApoC-II and apoC-III bound to the large emulsions as strongly as to the small emulsions.¹⁹⁰⁾ ApoC-II and apoC-III may prefer less positively curved surfaces (*i.e.*, large lipid particles) due to the convex hydrophobic face of apoC-II and the relatively large hydrophobic surface of amphipathic helices of apoC-III.²⁵⁹⁾

HL hydrolyzes lipoprotein-TG, but has a higher affinity for the smaller and denser LDL and HDL particles.³⁵⁾ HL works more effectively on the smaller, denser VLDL particles and least efficiently on CM.²⁶⁰⁾

The action of LPL is greater on CM than on VLDL.²⁸⁾ The susceptibility of large VLDL particles to hydrolysis by LPL is greater than that of the smaller VLDL.²⁶¹⁾ The preference of LPL for different lipoproteins seems to be directly related to the size of the particles. The weight fraction of TO in the emulsion surface monolayer of PC is independent of the relative concentration of TO in the particle or of the size of the particle.²⁶²⁾ Kinetic parameters of the LPL-mediated lipolysis activated by apoC-II indicate that LPL has similar apparent maximal activities in large and small emulsions. Binding parameters, dissociation constant and binding maximum, of apoC-II for large and small emulsions are similar.¹⁹⁰⁾ ApoE efficiently inhibited the LPL-mediated lipolysis of TG-rich emulsions *in vivo* and *in vitro*.¹⁴²⁾ Competition binding assays of different lipoproteins demonstrated that the binding affinity of CM to LPL was almost 50-fold higher than VLDL, and smaller hydrolyzed CM had less affinity than the larger CM, indicating a linear relation between CM size and binding affinity to LPL.²⁶³⁾ In the kinetic model for heterogeneous enzyme catalysis that includes LPL-apoC-II interactions, K_m^{app} is affected by the binding affinity of LPL to the lipid interface.¹⁸⁹⁾ Plasma apolipoproteins other than apoC-II are responsible for the regulation and inhibition of LPL action in a particle size-dependent manner.

I have prepared large (*ca.* 120 nm) and small (*ca.* 35 nm) emulsions consisting of TO and PC as the models of CM and remnants, respectively. Both large and small emulsions are hydrolyzed at similar rates in the presence of apoC-II.²⁶⁴⁾ ApoC-III and apoE work as LPL-inhibitors of the lipolysis activated by apoC-II.²⁶⁴⁾ The addition of apoC-III reduces the lipolytic velocities in both large and small emulsions.²⁶⁴⁾ The inhibition by apoE in small emulsions is more effective than for large emulsions.²⁶⁴⁾ ApoA-I inhibits the LPL-mediated lipolysis of small emulsions more effectively.²⁶⁴⁾ ApoA-I and apoE decrease both V_{max}^{app} and K_m^{app} as uncompetitive inhibitors, probably retarding the LPL-apoC-II-TO complex formation at

the emulsion surface monolayer.²⁶⁴⁾ On the other hand, apoC-III decreases $V_{\max}^{\text{app}}$ for both large and small emulsions and slightly increased K_m^{app} only for large emulsions, suggesting that apoC-III influences the lipolysis by inhibiting both formation of the enzyme-substrate complex and binding of LPL to emulsions.²⁶⁴⁾ ApoA-I in plasma may work as an intense inhibitory factor in the LPL reaction, especially for small particles. Normal human plasma contains sufficient amounts of apoA-I to inhibit the lipolytic activity of LPL. In human serum, the binding behaviors of apolipoproteins are very different between small and large emulsion particles.²⁶⁴⁾ The densities of apoC-II, apoC-III and apoE bound to large emulsion surfaces are greater than the respective values to small ones, while apoA-I more preferentially binds to small emulsion surfaces than large ones.²⁶⁴⁾ ApoA-I displaces apoC-II, apoC-III and apoE from small particle surfaces and plays a major role in regulating other apolipoprotein binding to the particles. The dissociation constant of apoA-I for the large emulsion particles is much greater than that for the small emulsion particles.¹⁹⁰⁾ The apoA-I binding capacities of lipid emulsions is correlated with the difference in the packing of surface layer¹⁹⁰⁾ and well explained in terms of the space available for accommodation of tandem repetitive class A amphipathic helical domains among PC head groups at the lipid particle surface.²⁶⁵⁾ In response to various lipid surfaces, apoA-I may be induced to undergo a conformational switch leading to a gross structural alteration of the protein.¹²⁴⁾ ApoA-I in plasma may regulate the lipolysis of TG-rich lipoproteins in a size-dependent manner, in addition to the well known inhibitory effects of apoC-III and apoE.

7. LIPOPROTEIN MODIFICATION

7.1. CM Remnants Postprandial hyperlipidemia is an independent risk factor for atherosclerosis.⁷⁾ There is a significant correlation between plasma apoB-48 levels and severity of coronary atherosclerosis.²⁶⁶⁾ CM undergoes the hydrolysis of TG by LPL in the capillary beds of peripheral tissue, and, as a result, CM remnants are produced.³⁴⁾ CM remnants generated in proximity to the artery wall during the postprandial period are atherogenic. The FC/TG ratio is increased by 17-fold in CM remnants compared with CM.²⁶⁷⁾ CM remnants are characterized by high FC content at the surface, which is different from LDL containing high amounts of CE in the core. CM remnants contain approximately 40-fold more FC per particle than LDL.²⁶⁸⁾ CM is too large to infiltrate into the vessel wall across the endothelial layer, but the diameter of CM remnants is small enough to penetrate the endothelium.⁴⁷⁾ CM remnants are rich in apoE, which plays a crucial role in lipoprotein metabolism. The major route of CM remnant uptake into macrophages occurs *via* LRP, while LDLR and scavenger receptors play minor roles.⁷³⁾ HSPGs participate in the remnant uptake by associating with LRP or acting alone as receptors.²⁶⁹⁾ Similar to scavenger receptors, HSPGs and LRP are not suppressed by intracellular cholesterol content.²⁷⁰⁾ CM remnants induce the accumulation of both TG and cholesterol in macrophages, and the rate of remnant uptake is influenced by the type of dietary fat in the particles.⁷³⁾ In striking contrast to LDL, the oxidation of CM remnants suppresses the induction of foam cell formation.⁷³⁾ CM remnants are cytotoxic to macrophages and smooth muscle cells.²⁷¹⁾ The

CM remnant-like particles downregulate the pro-inflammatory chemokine and cytokine secretion by monocytes and macrophages.^{272,273)}

To investigate the mechanisms of CM remnant-induced atherosclerosis, I have prepared FC-containing emulsion particles as a model for CM remnants. ApoE promotes the uptake of lipid emulsions by J774 macrophages.²⁴⁹⁾ The HSPG-LRP pathway plays an important role in the apoE-mediated emulsion uptake by J774 macrophages.²⁴⁹⁾ The uptake of FC-containing emulsions, but not LDL or acetylated LDL (acLDL), induced cytotoxicity to J774 macrophages.^{249,251)} The cytotoxicity induced by FC-containing emulsions is attenuated in the presence of heparin, indicating that the HSPG-LRP-mediated endocytosis of FC-rich particles induces a cytotoxic response.²⁵¹⁾ FC-containing emulsions increase the FC content of J774 macrophages.²⁵¹⁾ However, the cells incubated with FC-containing emulsions contain only a low level of CE.²⁵¹⁾ Unlike LDL, acLDL particles remarkably increase the CE content of the cells.²⁵¹⁾ AcLDL particles also increase the cellular FC to the level of FC-containing emulsion-loaded cells, but do not trigger cell death, indicating that the cytotoxicity is not correlated with the absolute levels of cellular FC.²⁵¹⁾ Cathepsin-L is the most active lysosomal protease in the degradation of intracellular or endocytosed protein substrates.^{274,275)} FC-containing emulsions induce the leakage of cathepsin-L to cytosol, which is not observed for acLDL.²⁵¹⁾ Upon lysosomal destabilization, cathepsins are released from lysosomes to cytosol.^{276–278)} Inhibition of the activity of cathepsin-L recovers the viability of macrophages that ingested FC-containing emulsions.²⁵¹⁾ These findings indicate that the treatment of FC-containing emulsions causes the lack of lysosomal stability. Cathepsins are known to promote cleavage of Bid, an apoptotic member of the Bcl-2 family, resulting in the release of cytochrome *c*, an initiating agent for apoptosis in mitochondria.^{279,280)} Cathepsins are implicated in atherosclerosis-based vascular disease.⁶³⁾ The expression of lysosomal cathepsin-L is increased in atherosclerotic plaques and correlated with apoptosis.²⁸¹⁾ FC-containing emulsions induce the activation of caspase-3.²⁵¹⁾ These findings indicate that FC-containing emulsion-induced apoptosis at least partially originates from the leakage of cathepsin-L. Considering that FC alters the physical properties of biological membranes, the uptake of FC-containing emulsions may cause FC enrichment in lysosomal membranes, resulting in their destabilization. CE is scarcely detected after the internalization of FC-containing emulsions, suggesting that FC from emulsion particles is not esterified by ACAT in the ER, due to the impairment of the FC transport from lysosomes to the ER mediated by NPC1 and NPC2. It is possible that NPC1 and NPC2 cannot efficiently transport the FC molecules rapidly accumulated in lysosomes without hydrolysis by lysosomal acid lipase, and that the direct incorporation of FC molecules into the lysosomal membranes and/or cholesterol crystallization cause lysosomal disruption and apoptosis.

7.2. SMase SMase hydrolyzes SM to phosphorylcholine and ceramide, a lipid second messenger in apoptosis, cell differentiation, and cell proliferation.²⁸²⁾ A variety of cell types present in atherosclerotic lesions secrete SMase.²⁸³⁾ Arterial wall SMase is involved in atherogenesis. SMase secretion by endothelial cells is stimulated by inflammatory cytokines, such as interleukin-1 β and interferon- γ .²⁸³⁾ LDL extracted

from human atherosclerotic lesions is highly enriched in SM compared with plasma LDL.²³⁰⁾ The ceramide content of aggregated LDL in atherosclerotic lesions is 10- to 50-fold higher than that of plasma LDL.²⁸⁴⁾ LDL isolated from human plasma possesses SMase activity.²⁸⁵⁾ In LDL aggregates, the SMase of LDL may catalyze the formation of ceramide in the contacting, adjacent lipoproteins.⁵⁰⁾ These LDL aggregates potentially induce macrophage foam cell formation.^{74,286)} The formation of ceramide from SM represents a critical step in atherosclerosis.

In emulsion particles, SMase hydrolyzes SM molecules, and yields ceramide molecules, which are retained in the particles, and water-soluble phosphorylcholine molecules, which are released.⁶⁰⁾ Although PC can act only as acceptors of hydrogen bonds, SM and ceramide can act as both acceptors and donors through their hydroxyl and amide group. Treatment of LDL particles with SMase induces both aggregation and fusion of the particles, which depend on the accumulation of ceramide within the particles.^{61,74,284)} I have shown that SMase promotes the aggregation and fusion of lipid emulsions depending on ceramide formation.^{287,288)} I have also confirmed the existence of ceramide-enriched domains in emulsion particles by confocal fluorescence microscopy²⁸⁸⁾ (Fig. 4). Two fluorescent lipid analogs distinguish the fluid and ordered membrane phases. DiI-C₁₈ predominantly partitions into the ordered membrane phases, whereas BODIPY-PC having a bulky moiety on an acyl chain favors the fluid phase.^{289–291)} The confocal images of ceramide-containing emulsions with two fluorescent probes have demonstrated the three-dimensional microdomains enriched in ceramide.²⁸⁸⁾ These ceramide-enriched domains present a variety of morphologies. In contrast, SM at the emulsion surface has no influence on the phase behavior.²⁸⁸⁾ SMase also induces the formation of ceramide-enriched domains extending from emulsion core to surface.²⁸⁸⁾ The SM-containing emulsion particles become larger after SMase treatment, demonstrating SMase leads to the fusion as well as the aggregation of emulsion particles.²⁸⁸⁾ Microdomain formation arises due to the properties of ceramide headgroup, and the large hydration of the phosphorylcholine group in SM may cause strong steric hindrance prohibiting hydrogen bonding in SM–SM interactions.²⁹²⁾ Ceramide molecules are likely to cluster and form three-dimensional, but not in-plane, microdomains in lipoprotein particles as well as emulsions. The ceramide-enriched domains may act as non-polar spots at the particle surface and lead initially to particle aggregation through hydrophobic associations between the domains in different particles.^{61,282)} Thus, a driving force for particle aggregation is the formation of ceramide-enriched domains. ApoE bound on the emulsion surface prevents the SMase-induced aggregation and fusion of the particles, but does not affect the SMase enzymatic activity.²⁸⁸⁾ Ceramide increases the amount of apoE bound to emulsion particles.²⁸⁸⁾ I have proposed apoE prefers to bind on ceramide-enriched domains exposed on particle surface, and thus inhibits the hydrophobic interaction between the particles followed by aggregation or fusion.²⁸⁸⁾

SMase modification of LDL facilitates their uptake by macrophages and foam cell formation.^{74,286)} In the presence of SMase, the endocytosis of LDL is enhanced and leads to an increase in LDL degradation and CE accumulation in J774 and mouse peritoneal macrophages.⁷⁴⁾ SMase-treated LDL is degraded at higher rates than native LDL in a variety

of cell lines.²⁹³⁾ LDL association with smooth muscle cells in the presence of SMase is greater than in the absence of SMase.²⁹⁴⁾ The uptake and degradation of lipoproteins from *apoE*-knockout mice by macrophages are dramatically increased by SMase, but neither LDLR nor scavenger receptors, CD36 or class A scavenger receptor, are involved in the process.²⁸⁶⁾ However, J774 macrophages internalize and degrade both matrix-retained and non-retained SMase-aggregated LDL, which are mediated partially by LRP.⁸¹⁾ The size of SMase-aggregated LDL (*ca.* 100 nm) is too small to elicit a phagocytic response.^{74,295)} SMase-treated aggregated and fused LDL bound to human aortic PGs more tightly in the affinity column than native LDL.^{54,61)} Furthermore, large amounts of ceramide ingested together with lipoproteins by macrophages possibly induce apoptosis. Therefore, elevation in plasma SM levels may result in the accumulation of ceramide in the arterial intima, leading to the development of atherosclerosis.

I have demonstrated that the generation of ceramide by SMase in emulsion particles increases their ability to be taken up by J774 macrophages without apolipoproteins.²⁸⁷⁾ The emulsion uptake is negatively correlated with the degree of particle aggregation by SMase.²⁸⁷⁾ The uptake of large SM-containing emulsions (*ca.* 230 nm) is much lower than that of normal-sized SM-containing emulsions (*ca.* 120 nm), indicating the uptake of the emulsion particles is largely dependent on their size.²⁸⁷⁾ The uptake of ceramide-containing emulsions is significantly larger than that of SM-containing emulsions.²⁸⁷⁾ Ceramide formed in the particles by SMase enhances their uptake into J774 macrophages, although the increase in size or particle aggregation decreases their uptake. HSPGs and LRP are crucial for the ceramide-enhanced emulsion uptake, because heparin or lactoferrin inhibit the emulsion uptake.²⁸⁷⁾ LRP is largely confined to clathrin-coated pits, which measure 180–240 nm in diameter.^{296,297)} It is likely that large particles are restricted to enter clathrin-coated pits, and thus the interaction of large particles with LRP is impaired. ApoE further increases the uptake of ceramide-containing emulsions compared with SM-containing emulsions, by increasing binding of apoE to ceramide-containing particles.^{287,288)} ApoE enhances the HSPG and LRP-mediated uptake of particles modified by SMase, not only due to the increased binding of apoE to ceramide-containing lipoproteins but also to the inhibition of particle aggregation. In the presence of LPL, ceramide-containing emulsions exhibit significantly higher uptake than SM-containing emulsions.²⁸⁷⁾ However, unlike apoE, LPL similarly enhances both SM-containing and ceramide-containing emulsion uptake.²⁸⁷⁾ From these findings, the generation of ceramide in lipoproteins by SMase may facilitate the macrophage uptake *via* HSPG and LRP pathways and play a crucial role in foam cell formation. Thus, ceramide may act as an important atherogenic molecule.

7.3. ApoB Denaturation In early atherosclerotic lesions, even before atheroma appears, symptomatic patients have significantly more arterial apoB deposits than patients without cardiovascular events. The modification of LDL leads to a conformational change in apoB. It has been proposed that the misfolding of apoB, its aggregation, resistance to proteolysis, and cytotoxicity are common motifs shared by modified LDL and amyloidogenic proteins.²⁹⁸⁾ The misfolded apoB that accumulated and aggregated in atherosclerotic lesions may induce cytotoxic effects.²⁹⁸⁾ The secondary structure and

conformation of apoB in LDL are imposed by lipid–protein interactions and dynamics.²⁹⁸⁾ Following an alteration in the water–lipid interface as a result of oxidation of lipids, the structure becomes destabilized and misfolded.²⁹⁸⁾ The secondary structure and conformation of apoB are severely altered in electronegative LDL (LDL(–)), a fraction of oxLDL isolated *in vivo*.²⁹⁹⁾ Decreased CD signal of apoB and shorter tryptophan fluorescence lifetime are observed for oxLDL, indicating that the secondary structure is changed, and that apoB unfolds into a conformation in which tryptophan residues are more exposed to water following oxidation.³⁰⁰⁾ The oxidation of LDL generates characteristic amyloid-like structures that are recognized by macrophages.³⁰¹⁾ The LDL(–) subfraction has strong immunoreactivity with an amyloid oligomer-specific antibody.³⁰²⁾ ApoB-100 in LDL(–) is misfolded, and this modification primes the aggregation of native LDL, similar to the protein amyloidogenesis.³⁰³⁾ During aggregation, a domino-style spread of apoB-100 misfolding from LDL(–) to all of the LDL particles occurs.³⁰³⁾ It has been predicted that a structure in the α_2 domain is highly prone to a conformational switch from a native α -helical arrangement toward cross- β aggregation with other protein molecules.³⁰³⁾ It is conceivable that apoB is dissociated partially or entirely from the LDL particle and is folded incorrectly as a result of several modifications in arterial walls, which may contribute to the development of atherosclerosis.

By small angle neutron scattering in combination with advanced shape reconstitution algorithms, a low resolution model of apoB has shown that the lipid-free protein adopts an expanded curved shape composed of distinct domains connected to flexible regions.³⁰⁴⁾ Sodium deoxycholate (NaDC) is a reasonable detergent to solubilize lipid-free apoB.³⁰⁵⁾ ApoB in NaDC has a mean length of 65 nm and appears to be long, thin, apparently flexible molecules.³⁰⁶⁾ I have shown that the CD spectrum of lipid-free apoB in NaDC is slightly different from that of apoB bound to LDL (LDL-apoB).³⁰⁷⁾ The analysis of the CD signal has revealed that the secondary structure of LDL-apoB is characterized by a large content of α -helix and smaller amount of β -sheet, β -turn and random coil.^{99,307)} The α -helix content of lipid-free apoB is lower than that of LDL-apoB, while the content of random coil is higher.³⁰⁷⁾ However, the overall secondary structure of lipid-free apoB is highly conserved, suggesting the solubilization of lipid-free apoB by NaDC may lead to only partial unfolding of the secondary structure in apoB rather than global protein unfolding as shown in oxLDL or LDL(–).^{299,300,303,307,308)}

I have investigated the cytotoxic effect of lipid-free apoB solubilized with NaDC, as a model for denatured apoB. Lipid-free apoB has cytotoxicity to J774 macrophages, HepG2 cells and Chinese hamster ovary cells, whereas LDL-apoB and lipid-free apoA-I have no effect on the cell viability.³⁰⁷⁾ Lipid-free apoB induces apoptosis in J774 macrophages assessed by caspase-3 activation and annexin V binding.³⁰⁷⁾ The uptake of lipid-free apoB by J774 macrophages is greater than that of LDL-apoB, indicating the uptake of apoB is promoted by the dissociation of apoB from LDL particles.³⁰⁷⁾ LDLR, HSPGs, and class A scavenger receptor are involved in the uptake of lipid-free apoB, but the uptake of lipid-free apoB is not involved in the cytotoxicity.³⁰⁷⁾ Lipid-free apoB, but not LDL-apoB or apoA-I, disrupts the lipid bilayer of large unilamellar vesicles containing calcein.³⁰⁷⁾ A lipid-free apoB induces

an increase in the intracellular Ca^{2+} concentration, which is brought about by influx of extracellular Ca^{2+} but not by calcium mobilization from intracellular Ca^{2+} stores.³⁰⁷⁾ Furthermore, the inhibitors for the uptake of lipid-free apoB do not show any inhibitory effects on the membrane disruption.³⁰⁷⁾ Taken together, lipid-free apoB is considered to induce cell death by disturbance of the plasma membrane. The high affinity of apoB for lipids may promote the binding of lipid-free apoB to the plasma membrane of the cells. Denatured apoB accumulated in atherosclerotic lesions may lead to cell death. In addition to other lipid components in modified LDL, apoB itself has an ability to induce apoptosis and plays a crucial role in the development of atherosclerotic lesions.

8. FUTURE DIRECTIONS

This review summarizes the current understanding of the metabolism of apoB-containing lipoproteins and the initiation of atherosclerosis. Numerous studies have revealed the properties of apolipoproteins and lipoprotein receptors, and have explored the mechanism of atherogenesis. Nevertheless, the detailed molecular mechanisms are not clearly understood. The interactions between different apolipoproteins on lipoprotein particles are largely unknown. In particular, the structure and functions of apoB are still only partly elucidated. Moreover, intracellular lipid transport and metabolism are extremely complex processes. A thorough understanding of the mechanisms involved in lipoprotein metabolism and atherogenesis will lead to the development of novel therapeutic strategies.

Conflict of Interest The author declares no conflict of interest.

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