# CHAPTER 22 Lipids and atherosclerosis

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# 1. Introduction

Atherosclerotic vascular disease is the cause of heart attacks, stroke, aortic aneurysms, and peripheral vascular disease, which together represent the most frequent causes of death in the industrialized world. Indeed, the aging of the population and the 'westernization' of world diet is predicted to increase the impact of atherosclerosis worldwide over the next few decades despite continuing advances in plasma lipid-lowering therapy (E. Braunwald, 1997).

Atherosclerosis progresses in a series of stages, although some lesions at each stage may not progress further or may even regress if inciting events, such as hypercholesterolemia, diabetes, smoking, or hypertension, are controlled [1-4] (Fig. 1). The initial



Fig. 1. Progression of atherosclerotic lesions. As noted in the text, only a portion of lesions at any stage progress and, under the proper conditions, lesion regression may occur.

stage involves the accumulation of subendothelial lipoproteins in focal areas of the arterial tree, usually at branch points with disturbed laminar flow. In response to this retention, a series of biological responses ensue, including (1) lipoprotein oxidation, (2) endothelial alterations, (3) inflammatory responses including T cell recruitment, cy-tokine secretion, monocyte chemotaxis, and subendothelial macrophage accumulation, (4) and intracellular cholesterol accumulation in macrophages. Much of the cholesterol is stored as cholesteryl fatty acid esters (CE) in cytoplasmic lipid droplets surrounded by a monolayer of phospholipid. These cytoplasmic droplets give the macrophages a foamy appearance when viewed by microscopy, and thus these cells are referred to as 'foam cells' (Fig. 2C). The presence of macrophage foam cells defines the earliest pathological lesion, referred to as the 'fatty streak'. Interestingly, fetuses of hypercholesterolemic mothers have been observed to have fatty streaks (W. Palinski, 1997). These fatty streaks disappear soon after birth, but virtually all Westerners have fatty streaks by the teenage years.

Although sensitive tests of endothelial function show abnormalities in vasodilatation in the very earliest phases of atherosclerosis (R.A. Vogel, 1998), fatty streaks are not occlusive and cause no overt symptoms. However, some fatty streaks may progress over years to more complex lesions that can give rise to chronic symptoms or, more importantly, acute events. An important event in the progression of fatty streaks involves the migration of smooth muscle cells from the media to the intima and the secretion of large amounts of collagen and other matrix proteins by these cells. In addition, macrophages proliferate and continue to accumulate more lipid. Smooth muscle cells can also accumulate lipid and become foam cells. These events give rise to so-called fibrous lesions, which are eccentric lesions consisting of lipid-loaded macrophages and smooth muscle cells covered by a fibrous cap. Further progression to complex lesions involves the accumulation of extracellular lipid, which results from a combination of aggregation and fusion of matrix-retained lipoproteins and release of lipid droplets from dying foam cells. Calcification, hemorrhage, and microthrombi can also be observed in these complex lesions [1].

At this stage, several fates of the lesion are possible [1,5,6]. Plasma cholesterol lowering can result in lesion regression, particularly of the foam cells. In this case, the macrophages begin to lose their cholesterol via the process of cholesterol efflux, and the number of macrophages decreases, probably through a combination of decreased monocyte entry, decreased macrophage proliferation, and increased macrophage egress and apoptosis. Alternatively, the complex lesions can progress. If arterial occlusion increases gradually, the patient may experience exercise-induced ischemia, but collateral vessel formation often prevents additional clinical symptoms. However, if the lesions rupture or erode before they become large and occlusive, acute vascular events such as unstable angina, heart attacks, sudden death, or strokes can occur. Rupture involves the abrupt disruption of the fibrous cap, followed by exposure of thrombogenic material and acute thrombosis. Importantly, rupture mostly occurs in lipid-rich and macrophagerich 'shoulder' regions of the plaque and is probably triggered by the degradation of the fibrous cap by proteases secreted by macrophages or released from dying foam cells. Physical stresses related to pools of soft lipid underneath a thin fibrous cap also contribute to plaque rupture. These pools of lipid and cellular debris, often referred



Fig. 2. Lipoprotein aggregation and macrophage foam cell formation. (A) Freeze-etch replica-plated electron micrograph of rabbit aorta subendothelium 2 hours after intravenous injection of LDL (from Nievelstein et al. (1991) Arterio. Thromb. 11: 1795–1805). (B) A J774 murine macrophage (M) immediately after plating on sphingomyelinase-induced LDL aggregates (arrow) formed on the surface matrix of smooth muscle cells (S). (C) After 24 hours of incubation, the aggregates have been internalized by the macrophage, which now has large cytoplasmic neutral lipid droplets consisting mostly of cholesteryl ester (arrow). The cytoplasmic droplets are characteristic of lesional foam cells. Bars in B and C, 1  $\mu$ m. Panels B and C are from Tabas et al. (1993) J. Biol. Chem. 268: 20419–20432.

to as 'necrotic' or 'lipid' cores, result from the death of macrophage foam cells (M.J. Mitchinson, 1995).

As is evident from this overview, lipids are the sine qua non of atherosclerosis. Indeed, the 'athero' of 'atherosclerosis' is derived from the Greek word for 'gruel',

Lipid	Fatty streak	Intermediate lesion	Fibrous lesion	Advanced lesion
Cholesterol	9.6	21.1	22.5	31.5
Triglyceride	2.8	4.4	5.2	6.0
Cholesteryl ester	77.0	55.0	55.5	47.2
Phospholipid	10.1	19.6	16.8	15.3
Phosphatidylcholine	4.8	7.6	4.5	4.3
Sphingomyelin	5.6	11.0	11.7	10.1
Lysophosphatidylcholine	0.3	1.0	0.6	0.9

Table 1
Percent weight of major lesion lipids in four progressive stages of atherosclerotic lesions

Adapted from Katz et al. [16].

which refers to the massive accumulation of lipids in these vascular lesions. The major types of lipids that accumulate during the various stages of atherosclerosis are shown in Table 1. In addition, there are many lipids that are minor in quantity but, because of their biological activities, are thought to have a major impact on atherogenesis. This chapter will cover the properties and activities of many of the lipids that occur in atherosclerotic lesions, with an emphasis on their roles in lesion development and progression.

# 2. Cholesterol and atherosclerosis

### 2.1. Cholesterol deposition in the arterial wall

As alluded to in the Introduction, the primary event in atherogenesis is cholesterol deposition in the arterial wall. The cholesterol originates from circulating plasma lipoproteins, which contain both unesterified cholesterol ('free' cholesterol, or FC) and cholesteryl ester (CE) (see Chapter 18). The two classes of lipoproteins that contribute most to atherogenesis are low density lipoprotein (LDL) and so-called remnant lipoproteins, which are the lipolytic products of chylomicrons and very low density lipoprotein (VLDL). Plasma lipoproteins continually enter the subendothelial space of vessels via 'leakage' through transient gaps between endothelial cells and probably also via endothelial transcytosis. Under normal conditions, lipoproteins are not retained in the subendothelium and simply re-enter the circulation. In certain focal areas of the arterial tree, however, lipoprotein retention by subendothelial extracellular matrix is increased, leading to their net accumulation in the arterial wall. This retained material elicits a series of biological responses, leading to the cellular and extracellular processes that constitute atherosclerotic lesion formation ([4,7]; see also Section 1). Because a high concentration of circulating atherogenic lipoproteins promotes the accumulation of these lipoproteins in the arterial wall, this model explains the well-established relationship between plasma cholesterol levels and atherosclerosis in both experimental animal models and humans.

The fate of the FC and CE moieties of retained lipoproteins includes both extracellular and intracellular processes. Extracellular matrix-retained lipoproteins are modified by lipases, proteases, and oxidation reactions (P.T. Kovanen, 2000) [8]. These reactions can lead to the generation of lipid vesicles that are rich in FC but poor in protein and CE (H. Kruth, 1985). The biological or pathological significance of these FC-rich vesicles is not known. Other reactions lead to the generation of modified lipoproteins that act as extracellular signaling molecules on lesional cells or that are avidly internalized by macrophages and smooth muscle cells. Thus, these modified lipoproteins are responsible for foam cell formation and a variety of cell signaling events.

### 2.2. Cholesterol accumulation in lesional macrophages: lipoprotein internalization

The major cell type that internalizes subendothelial lipoproteins is the macrophage [2,3]. Lesional macrophages are derived from circulating monocytes that enter the arterial wall in response to chemokines; the chemokines are secreted by endothelial cells in response to both underlying retained lipoproteins and T cell-derived cytokines. Under the influence of other molecules secreted by endothelial cells, notably macrophage colony stimulating factor, subendothelial monocytes differentiate into macrophages. The differentiated macrophages then engage and internalize subendothelial lipoproteins and thus accumulate lipoprotein-derived cholesterol in the form of intracellular cholesteryl ester droplets (foam cell formation). As outlined in the Section 1, this cellular event is the hallmark of early lesion development and also contributes to late lesional complications.

Two key issues in the area of macrophage foam cell formation include the cell-surface processes and receptors involved in lipoprotein internalization and the metabolic fate of lipoprotein-derived cholesterol following internalization [9]. Most studies examining macrophage–lipoprotein interactions use an experimental system in which monolayers of cultured macrophages are incubated with soluble, monomeric lipoproteins dissolved in tissue culture media. These studies have revealed that native LDL is poorly internalized by macrophages, suggesting that LDL undergoes modification in the arterial wall. While a variety of LDL modifications have been proposed, two types, namely oxidation and aggregation, have received the most attention [8,10,11].

LDL particles with oxidative modifications of both its protein and lipid moieties are known to exist in atherosclerotic lesions and are readily internalized by macrophages. A number of receptors have been implicated in oxidized LDL uptake by macrophages, including class A and B scavenger receptors (e.g., CD36) and lectin-like oxidized LDL receptor-1 (LOX-1) (Chapter 21). While internalization of oxidized LDL by macrophages may have important implications in atherogenesis, it is unlikely that all of the hallmarks of macrophage intracellular cholesterol metabolism that are known occur in lesions can be explained by this process alone [8].

As stated above, lipoproteins in the subendothelium are also known to be aggregated and fused, which may result from oxidation, lipolysis, or proteolysis [8,12] (Fig. 2A). For example, hydrolysis of the sphingomyelin on LDL particles to ceramide by sphingomyelinase leads to LDL aggregates that appear similar to those that exist in lesions, and there is evidence that LDL in the arterial wall is hydrolyzed by a form of sphingomyelinase secreted by arterial-wall cells [13] (Fig. 2B). Aggregated lipoproteins, like oxidized LDL, are readily internalized by macrophages. When aggregated LDL is added in tissue culture medium to monolayers of cultured macrophages, the LDL receptor (Chapter 21) seems to participate in a phagocytic-like process to internalize these particles. In vivo, however, most of the aggregated lipoproteins are bound to extracellular matrix, and newer experimental systems that attempt to mimic the uptake of retained and aggregated LDL have revealed that multiple receptors in addition to or instead of the LDL receptor are involved. Most importantly, macrophage internalization of aggregated lipoproteins leads to massive CE accumulation, which is the key intracellular cholesterol metabolic event that is known to occur in macrophages in lesions [9] (Fig. 2C).

Remnant lipoproteins are also important in atherogenesis (R.W. Mahley, 1985; R.J. Havel, 2000). These particles can be internalized by macrophages in their native form, although both oxidation and aggregation of these particles occur and probably further enhance macrophage uptake. The receptor or receptors involved in the uptake of remnant particles is not definitively known, but the likely candidates are the LDL receptor and the LDL receptor-related protein (LRP), which interact with the apolipoprotein E moiety of the remnant lipoproteins (Chapter 21). Remnant lipoproteins, like aggregated lipoproteins, lead to massive cholesteryl ester accumulation in macrophages. Finally, it is worth mentioning that another lipoprotein called lipoprotein(a), in which a large glycoprotein called apolipoprotein(a) is covalently attached to the apolipoprotein B100 moiety of LDL, has been implicated in atherogenesis (A.M. Scanu, 1998). Although macrophage receptors for lipoprotein(a) have been described, neither the mechanism of atherogeneicity nor the role of lipoprotein(a) lipids in macrophage cholesterol loading and lesion development are known.

# 2.3. Cholesterol accumulation in lesional macrophages: intracellular trafficking of lipoprotein-derived cholesterol

The fate of lipoprotein cholesterol after internalization is a key issue in understanding the biology and pathology of lesional macrophages. After internalization by receptormediated endocytosis or phagocytosis, the lipoproteins are delivered to late endosomes or lysosomes, where hydrolysis of proteins and lipids occurs. Most importantly, the large lipoprotein-CE stores are hydrolyzed by a lysosomal enzyme called lysosomal acid lipase. The liberated FC then trafficks to the plasma membrane and other cellular sites [9].

The trafficking of lipoprotein-derived cholesterol from lysosomes has been a major area of focus in the field of intracellular cholesterol metabolism, and many of the cellular and molecular events are not known (Chapter 17). By analyzing cells with mutations in cholesterol transport, investigators have identified roles for two proteins, called npc1 and npc2 (HE1), in lysosomal and/or endosomal cholesterol transport (E.J. Blanchette-Mackie, 2000; P. Lobel, 2000). In addition, the lipid lysobisphosphatidic acid may also play a role in these processes (J. Gruenberg, 1999). The mechanisms by which these molecules are involved in cholesterol transport, however, are poorly understood. One current model suggests that there is an initial npc1-independent phase consisting of rapid cholesterol transport from late endosomes or lysosomes to the plasma membrane, probably by vesicular transport (T.Y. Chang, 2000; Y. Lange, 2000).

According to this model, the cholesterol is then internalized into a 'sorting organelle', from which cholesterol is distributed to peripheral cellular sites in an npc1-dependent manner. This transport process probably also occurs via vesicular transport. It must be emphasized, however, that until the mechanism of action of the molecules mentioned above and other molecules are elucidated and the cellular sites in the itinerary identified, this model must be considered hypothetical. It is also likely that different cell types and different conditions in the same cell type may result in different cholesterol trafficking patterns.

From the point of view of atherosclerosis, the two most important peripheral trafficking pathways are those to the endoplasmic reticulum, where cholesterol is esterified by acyl-CoA : cholesterol acyltransferase (ACAT), and to the plasma membrane, where cholesterol can be transferred to extracellular acceptors in a process known as cholesterol efflux (Chapter 20). The former process leads to the massive cholesteryl ester accumulation seen in foam cells [9,14,15]. The ACAT reaction utilizes primarily oleoyl-CoA, and so ACAT-derived CE is rich in oleate. In contrast, plasma lipoprotein-CE tends to be rich in linoleate. As expected, therefore, the cholesteryl oleate : cholesteryl linoleate ratio in foam cell-rich fatty streak lesions is relatively high (1.9) [16]. However, the ratio in advanced lesions is only 1.1, suggesting an increase in lipoprotein-CE in advanced atheromata due to poor cellular uptake of lipoproteins or to defective lysosomal hydrolysis following uptake by lesional cells. Further discussion of the cholesterol esterification pathway appears in Chapter 15, and cholesterol efflux, which is an important mechanism that may prevent or reverse foam cell formation, is covered in Chapter 20.

### 2.4. Accumulation of free cholesterol in lesional macrophages

Interestingly, foam cells in advanced atherosclerotic lesions accumulate large amounts of FC [16], some of which is in crystalline form and may be deposited in the extracellular space when foam cells die (Fig. 3). For example, while 2 of 13 abdominal aortic and femoral artery fatty streak lesions contained cholesterol crystals, all of 24 advanced lesions had these structures [16]. The mechanism of FC accumulation is not known, but could involve either defects in cholesterol trafficking to ACAT or a decrease in ACAT activity itself. Because much of the FC accumulating in the cells appears to be associated with lysosomes, it is tempting to speculate that defects in lysosomal cholesterol transport arise in advanced foam cells. In this context, macrophages exposed to oxidized LDL can internalize a substantial amount of cholesterol, but there is relatively little stimulation of ACAT-mediated cholesterol esterification [8]. According to one model, oxysterol-induced inhibition of lysosomal sphingomyelinase leads to accumulation of lysosomal sphingomyelin, which binds cholesterol and thus inhibits transport of the cholesterol out of lysosomes (M. Aviram, 1995).

Free cholesterol accumulation in macrophages may be an important cause of macrophage death in advanced atherosclerotic lesions [17] (Fig. 4). Death induced by intracellular free cholesterol excess probably involves both necrosis and apoptosis. Necrotic death may result from the malfunction of critical plasma membrane proteins exposed to a microenvironment with a high cholesterol : phospholipid ratio. Intracellular cholesterol crystal accumulation may also contribute to this form of death.



Fig. 3. (A) Intracellular free cholesterol accumulation in a lesional foam cell. Electron micrograph of the cytoplasm of a foam cell isolated from an advanced aortic atherosclerotic lesion in a cholesterol-fed rabbit. The cell was treated with filipin, which forms spicules with unesterified cholesterol. Multiple spicules are observed in vesicles, shown to be lysosomes (depicted by arrows). Bar, 0.5  $\mu$ m. (From Shio et al. (1979) Lab. Invest. 41: 160–167.) (B) Extracellular cholesterol crystals in an advanced atherosclerotic lesion. The section is from the proximal aorta of a fat-fed apolipoprotein E knockout mouse. This mouse model is often used to study atherosclerosis in vivo because the high plasma levels of remnant lipoproteins resulting from absence of apolipoprotein E leads to a much greater degree of atherosclerosis lesion development than observed in wild-type mice. The arrows depict the areas of cholesterol crystals.



Fig. 4. FC-induced death in macrophages. Cultured macrophages were cultured under control conditions (A) or conditions leading primarily to CE loading (B) or FC loading (C). Note that FC-loaded macrophages, but not those loaded mostly with CE, have obvious signs of cytotoxicity, including detachment from the dish and altered morphology. (From Tabas et al. (1996) J. Biol. Chem. 271: 22773–22781.)

Free cholesterol-induced apoptosis in cultured macrophages involves both activation of Fas ligand and release of cytochrome c from mitochondria, which is associated with increased levels of Bax in these cells (I. Tabas, 2000 and 2001).

### 2.5. Cholesterol accumulation in lesional smooth muscle cells

Smooth muscle cells in atherosclerotic lesions also accumulate large amounts of cholesteryl ester, although the mechanisms involved are poorly understood [18]. As with macrophages, native LDL is a poor inducer of foam cell formation, but substantial cholesterol accumulation has been induced in cultured smooth muscle cells by aggregated LDL (L. Badimon, 1998). Cytokine treatment of cultured smooth muscle cells leads to the induction of the type A scavenger receptor, but there are no data specifically showing that oxidized LDL can cause foam cell formation in smooth muscle cells either in vitro or in vivo. Finally, remnant lipoproteins, including  $\beta$ -VLDL, cationized LDL, and cholesteryl ester emulsions, can induce cholesterol accumulation in cultured smooth muscle cells, but their roles in vivo are not known.

# 2.6. The fate of foam cell cholesterol in atheromata

Cholesteryl esters, which exist in membrane-bound droplets in the macrophage cytoplasm, undergo a continuous cycle of hydrolysis by neutral cholesteryl ester hydrolase and re-esterification by ACAT (M.S. Brown and J.L. Goldstein, 1980). If extracellular cholesterol acceptors, like HDL or apolipoprotein A-I, are available, some of this cholesterol can leave the cell, enter the circulation, and be transported to the liver in a process known as reverse cholesterol transport (Chapter 20). The fatty acyl and neutral lipid composition of foam cell droplets may influence this process by affecting the fluidity of the droplets. It is also possible that the foam cells themselves can leave lesions, although this event has been difficult to document. Finally, as described above, foam cells in atherosclerotic lesions die, and thus cellular stores of cholesteryl ester and free cholesterol, including cholesterol crystals, can be released into the lesions. This process undoubtedly contributes to the formation of the necrotic, or lipid, core of advanced atheromata, because such areas contain macrophage debris (M.J. Mitchinson, 1995). As described in the Introduction, necrotic cores have important pathophysiologic significance because they predispose lesions to plaque rupture, the proximate cause of acute vascular clinical syndromes.

# 3. Oxysterols and atherosclerosis

### 3.1. Origins of oxysterols

Oxysterols arise from dietary sources, non-enzymatic oxidation, and enzymatic oxidation reactions [19]. The structure of some of the oxysterols that may be involved in atherosclerosis are shown in Fig. 5. Dietary oxysterols are incorporated into chylomicrons and include 7-ketocholesterol (7K),  $7\alpha$ - and  $7\beta$ -hydroxycholesterol (7OH), and  $\alpha$ - and  $\beta$ -5,6-epoxycholesterol (EPOX). 7OH, 7K, 24-hydroxycholesterol (24OH), 25hydroxycholesterol (25OH), and 27-hydroxycholesterol (27OH) can be formed in vivo, but it is not clear whether non-enzymatic or enzymatic mechanism are involved. Specific enzymatic reactions include the formation of  $7\alpha$ OH by cholesterol  $7\alpha$ -hydroxylase in liver (Chapter 16) and 27OH and 3 $\beta$ -hydroxy-5-cholestenoic acid by 27-hydroxylase in liver and macrophages.



Fig. 5. Structures of some oxysterols that have been implicated in atherogenesis. (Adapted from Brown and Jessup [19].)

### 3.2. Oxysterols in plasma, lipoproteins, and atherosclerotic lesions

The most abundant oxysterols in human plasma are 27OH, 24OH, and 7 $\alpha$ OH (Fig. 5), and most of these are esterified to fatty acids at the 3 $\beta$  position by lecithin : cholesterol acyltransferase [19]. Both free and esterified oxysterols partition in lipoproteins similar to cholesterol and cholesteryl ester, respectively, although 27OH is generally not found in VLDL, and unesterified 25OH can be associated with albumin. With the possible exception of 7 $\beta$ OH, there is no clear relationship between plasma levels of oxysterols and atherosclerosis.

Oxysterols are also found in copper-oxidized LDL and consist predominantly of 7K, 7-hydroperoxy-cholesterol (70OH), 7OH, and EPOX. LDL oxidized by more physiologic means, such as during contact with macrophages or by incubation with lipoxygenase, accumulates 7OOH (Fig. 5). Myeloperoxidase-treated cholesterol leads to the formation of unique chlorinated cholesterol derivatives, which can give rise to cholesterol epoxides [19].

The predominant oxysterols in atherosclerotic lesions include 27OH and 7K, where the levels are approximately 1% of cholesterol, with smaller amounts of 7OH; as in plasma, the vast majority are in the esterified form [19]. These oxysterols are found mostly in macrophage foam cells, which probably reflects the abundance of 27OH (Section 3.3). Moreover, 27-hydroxylated 7K is found in human atherosclerotic lesions, probably via macrophage sterol 27-hydroxylase acting on 7K internalized by macrophages (W. Jessup, 2000). When cultured macrophages are incubated with copper-oxidized LDL, 50% of the accumulated sterols are oxysterols, many of which accumulate in lysosomes as non-ACAT-derived oxidized fatty acid esters of oxysterols (W. Jessup, 2000). Although copper-oxidized LDL contains substantial amounts of 7OOH, macrophages accumulate very little of this lipid probably due to conversion to 7OH by phospholipid hydroperoxide glutathione peroxidase [19].

### 3.3. Physiologic significance of oxysterols in atherosclerosis

The proposed roles of oxysterols in atherosclerosis are based primarily on the results of cell-culture experiments. There are a number of in vivo studies in which investigators have exposed animals to oxysterols through diet or injection, but the overall results are not conclusive. For example, in a review by Brown and Jessup of 13 oxysterol dietary studies, six showed an increase in atherosclerosis, but four demonstrated a decrease in lesion size and three showed no effect [19]. Differences in animal models and types and doses of oxysterols undoubtedly account for some of these differences.

There are four major areas of oxysterol biology that have emerged from cell-culture studies. These include oxysterol effects on the regulation of intracellular cholesterol metabolism, cellular cytotoxicity, sterol efflux from macrophages, and activation of nuclear transcription factors. Issues related to oxysterols and intracellular cholesterol metabolism are covered in detail in Chapters 15 and 16. In brief, both cholesterol and certain oxysterols, such as 25OH and 7K, suppress the proteolytic activation of sterol response element binding protein. This, in turn, leads to transcriptional down-regulation of the LDL receptor and certain enzymes in the cholesterol biosynthetic

and fatty acid synthesis and metabolism pathways. 25OH and 7K, like cholesterol, can also promote the degradation of 3-hydroxyglutaryl-3-methylglutaryl CoA reductase, a rate-limiting enzyme in isoprenoid and cholesterol biosynthesis, and 25OH can activate ACAT and suppress neutral cholesteryl ester hydrolase activity. Cells possess a protein that binds oxysterols, called oxysterol binding protein, but its role in cellular responses to oxysterols is not known. Importantly, the physiologic role of oxysterols in cellular cholesterol metabolism as it relates to atherosclerosis is far from certain, particularly because the concentrations of oxysterols used in most macrophage cell-culture studies far exceeds those found in macrophage foam cells in vivo [19].

Oxysterols have diverse roles in cholesterol efflux, a critical topic in foam cell biology. On the one hand, cells incubated with 7K and 25OH have decreased cholesterol efflux. Possible mechanisms include inhibition of membrane desorption of cholesterol or phospholipids or, as mentioned above, inhibition of lysosomal sphingomyelinase leading to lysosomal sequestration of cholesterol (M. Aviram, 1995). On the other hand, the conversion of cholesterol by macrophage sterol 27-hydroxylase to 27OH and  $3\beta$ -hydroxy-5-cholestenoic acid, which are efficiently effluxed from cells, has been proposed to promote sterol efflux from foam cells (I. Björkhem, 1994). Indeed, 27OH is a prominent oxysterol found in lesions, and some studies have shown an inverse correlation between 27-hydroxylase levels and atherosclerosis (N.R. Cary, 2001). Future studies with 27-hydroxylase-deficient mice in general, and mice with a macrophage-specific deficiency of 27-hydroxylase in particular, should shed further light on the physiologic importance of this pathway.

A major area of investigation has been on the cytotoxic effects of certain oxysterols on cultured endothelial cells, macrophages, and smooth muscle cells [19]. The most potent cytotoxic oxysterols include 27OH, 7 $\beta$ OOH, 7 $\alpha$ OH, and 7K (G.M. Chisolm, 1996). These sterols damage cells through a variety of mechanisms including cholesterol starvation, membrane perturbation, cellular lipid peroxidation, and activation of apoptotic pathways. Although death of endothelial cells, macrophages, and smooth muscle cells does occur in atherosclerotic lesions and might be expected to promote lesion development and complications, the role of isolated oxysterols or oxysterols in oxidized LDL in these events is far from certain. The conditions used in many cell-culture studies, notably high concentrations of oxysterols and/or serum-free medium, may not reflect the situation in vivo.

Exciting recent work has revealed that certain oxysterols are activators of nuclear transcription factors [20]. In particular, four oxysterols found in vivo, 24,25 EPOX, 24OH, 22OH, and 27OH, but not cholesterol, activate LXR $\alpha$ , LXR $\beta$ , and FXR (Chapter 16). Once activated, these molecules heterodimerize with activated RXR, forming active transcription factors which translocate to the nucleus and induce several genes important in atherosclerosis. In particular, a set of genes important in the reverse cholesterol transport pathway is activated by this pathway [21]. The proteins encoded by these genes include (1) macrophage ABCA1 and apolipoprotein E, which promote cholesterol efflux from foam cells, (2) plasma cholesteryl ester transfer protein, which transfers HDL-cholesterol to lipoproteins that can be internalized by hepatocytes, (3) and liver cholesterol  $7\alpha$ -hydroxylase, which is the key enzyme that converts hepatocyte cholesterol into bile acids for excretion. Recent studies with genetically manipulated

mice have demonstrated the importance of the LXR pathway in vivo. For example, activation of RXR reduces atherosclerosis in apolipoprotein E knockout mice (J. Auwerx, 2001), and the livers of cholesterol-fed LXR $\alpha$  knockout mice accumulate very large amounts of cholesterol (D.J. Mangelsdorf, 1998).

In summary, oxysterols are known to exist in atherosclerotic lesions and have been demonstrated in cell-culture experiments to have profound cellular effects that could influence the development, progression, and reversal of atherosclerosis. The key question in this field of research, however, is whether the concentrations of oxysterols in vivo are high enough to influence atherogenesis. Thus far, only the oxysterol-activated nuclear transcription pathway has been directly supported by in vivo data, and even in this case the precise roles and identification of the activating oxysterols in vivo have not yet been elucidated.

# 4. Triglycerides and atherosclerosis

There are two major issues that arise when considering the role of triglycerides in atherosclerosis: the effect of triglyceride-containing lipoproteins in the plasma on atherosclerotic lesion development, and the direct role of arterial-wall triglycerides in atherogenesis. The association between triglyceride-rich lipoproteins and atherosclerotic vascular disease is often difficult to assess due to complex metabolic relationships between these lipoproteins and other risk factors for atherosclerosis, including low plasma HDL and hyperfibrinogenemia [22]. Certainly, those triglyceride-containing lipoproteins that also have a high content of cholesterol, such as remnant lipoproteins, have been shown to be associated with atherosclerotic disease and probably function largely by delivering large amounts of cholesterol to the subendothelial space. The possibility that triglycerides and triglyceride-derived fatty acids also contribute to the atherogenicity of these lipoproteins, however, must also be considered (Section 5). Interestingly, metabolic disorders resulting in severe increases in plasma triglyceride, such as lipoprotein lipase deficiency, are not associated with increased risk of atherosclerosis. In these disorders, the triglyceride-rich lipoproteins are so large that they cannot enter the arterial wall (D.B. Zilversmit, 1989).

Triglycerides constitute a measurable proportion of the lipid content of atherosclerotic lesions, although considerably less than that of cholesterol. In one study, for example, the weight-percentages of triglycerides in fatty streaks and advanced lipid-rich lesions were 2.8 and 6.0, respectively; the corresponding total cholesterol percentages were 9.6 and 31.5, respectively [16]. However, both extracellular and intracellular triglycerides could play an important role in atherogenesis by serving as a source of free fatty acids following hydrolysis by extracellular and intracellular lipases. Indeed, the relatively low content of lesional triglyceride may be partially due to this process. As discussed in Section 5, free fatty acids are precursors of potentially important bioactive lipids.

Lesional cells in general, and macrophages in particular, can accumulate triglycerides via the uptake of triglyceride-rich lipoproteins and by intracellular triglyceride synthesis. Although triglycerides are a relatively minor component of neutral lipid droplets in lesional foam cells, even small percentages can lower the melting temperature of these

droplets [16]. Polyunsaturated fatty acids in the cholesteryl esters and triglycerides of lipid droplets also lower their melting temperature. Liquid neutral lipid droplets in foam cells are hydrolyzed at a more rapid rate than liquid crystalline droplets, and thus foam cells with a higher content of triglycerides may have an increased rate of cholesterol efflux (J.M. Glick, 1989).

# 5. Fatty acids and atherosclerosis

# 5.1. Direct effects of fatty acids

Fatty acids may have direct effects on atherogenesis and are also the precursors of specific bioactive lipids that may have important roles in lesion development. Lesions contain up to 0.4 mg of free fatty acids per gram of wet tissue (L. Robert, 1976). In terms of direct effects, high concentrations of extracellular free fatty acids could, in theory, lower the pH of focal areas in lesions, thus enabling the action of certain enzymes, such as lysosomal hydrolases that are secreted or leak from cells. When taken up by cells, fatty acids stimulate cholesteryl ester, phospholipid, and triglyceride syntheses. Individual types of fatty acids can have specific effects. For example, oleate, but not linoleate, is a potent stimulator of the ACAT reaction [15], and neutral lipids esterified to polyunsaturated fatty acids have a lower melting temperature, which tends to promote neutral lipid hydrolysis and lipid efflux.

# 5.2. Oxidation of long-chain polyunsaturated fatty acids: introduction

The major bioactive products of free fatty acid metabolism relevant to atherosclerosis are those that result from enzymatic or non-enzymatic oxidation of polyunsaturated long chain fatty acids. In most cases, these fatty acids are derived from phospholipase  $A_2$ -mediated hydrolysis of phospholipids (Chapter 11) in cellular membranes or lipoproteins, or from lysosomal hydrolysis of lipoproteins after internalization by lesional cells. In particular, arachidonic acid is released from cellular membrane phospholipids by arachidonic acid-selective cytosolic phospholipase  $A_2$ . In addition, there is evidence that group II secretory phospholipases  $A_2$  (Chapter 11) hydrolyzes extracellular lesional lipoproteins, and lysosomal phospholipases and cholesterol esterase release fatty acids from the phospholipids and cholesteryl esters of internalized lipoproteins. Indeed, Goldstein and Brown recently surmised that at least one aspect of the atherogenicity of LDL may lie in its ability to deliver unsaturated fatty acids, in the from of phospholipids and cholesteryl esters, to lesions (J.L. Goldstein and M.S. Brown, 2001).

# 5.3. Oxidative metabolites of arachidonic acid

An important fate of arachidonic acid is enzymatic conversion to prostaglandins by one of two prostaglandin G/H synthases [23]. As described Chapter 13, these enzymes have both cyclooxygenase and hydroperoxidase activities, and are often referred to as COX-1 and COX-2. Although atherosclerotic lesions express both isoforms, mature



Fig. 6. Structures of two arachidonic acid derivatives proposed to play important roles in thrombosis and atherogenesis. Thromboxane  $A_2$  is a potent inducer of platelet aggregation that contributes to acute thrombosis in advanced atherosclerosis in vivo. The isoprostane 8-*iso*-PGF<sub>2α</sub> is being investigated as a marker of oxidative stress in atherosclerosis and may also have direct atherogenic effects on platelets and smooth muscle cells.

human platelets express only COX-1. In this regard, the most well-documented role of a cyclooxygenase product on atherothrombotic vascular disease is platelet-derived thromboxane  $A_2$  (Fig. 6). Thromboxane  $A_2$  is a potent inducer of platelet aggregation and vasoconstriction, and aspirin-induced inhibition of platelet COX-1 accounts for its benefit in the secondary prevention of strokes and myocardial infarction. The roles of other prostaglandins synthesized in lesions are uncertain. For example, vascular endothelial cells synthesize prostacyclin, which blocks platelet aggregation, cellular interactions, and vascular smooth muscle cell proliferation in vitro and in vivo. However, there is no evidence using drug-induced inhibition of prostacyclin synthesis or prostacyclin receptor-deficient mice that prostacyclin has a net effect on atherosclerotic lesion development in vivo (G.A. Fitzgerald, 2001). A third COX-derived prostaglandin that has received recent interest is 15-deoxy-D12,14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), which, at least in vitro, is an agonist of peroxisomal proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) (C.K. Glass, 1998). PPARy is expressed in atherosclerotic lesions and in cultured endothelial cells, vascular smooth muscle cells, and monocyte/macrophages (B. Staels, 2000). Although cell-culture studies have revealed a variety of biological effects that may be pro- or anti-atherogenic, recent in vivo studies suggest that PPAR $\gamma$  plays an anti-atherogenic role (C.K. Glass, 2000). The physiologic significance of 15d-PGJ<sub>2</sub>, which also acts on  $I\kappa B$  kinase, in PPAR $\gamma$  biology remains to be determined.

A relatively new class of oxidized arachidonic acid derivatives with potential relevance to atherosclerosis are  $F_2$  isoprostanes [24] (Fig. 6). These compounds form as a result of non-enzymatic, free-radical attack of the fatty acid moieties of cellular or lipoprotein phospholipids, followed by release of the isoprostanes from the phospho-

lipids by a phospholipase. 8-*iso*-PGF<sub>2</sub> may also be formed by the action of COX-1 or COX-2 in platelets or monocytes, respectively, but the significance of COX-dependent 8-*iso*-PGF<sub>2</sub> formation in vivo is unproven. F<sub>2</sub> isoprostanes circulate in the plasma and appear in the urine as free compounds or esterified to phospholipids, and 8-*iso*-PGF<sub>2</sub> is found in atherosclerotic lesions in association with macrophages and smooth muscle cells. The potential significance of isoprostanes to atherosclerosis are their effects on platelets and vascular cells, as demonstrated in cell-culture studies, and their potential usefulness as a non-invasive marker of oxidant stress. 8-*iso*-PGF<sub>2</sub> induces platelet aggregation, DNA synthesis in vascular smooth muscle cells, and vasoconstriction. Moreover, elevated levels of  $F_2$  isoprostanes are found in cigarette smokers, diabetics, and subjects with hypercholesterolemia, where they may serve as an indicator of increased lipid peroxidation.

Arachidonic acid can also be oxidized by 5-, 12-, and/or 15-lipoxygenases to various mono-, di-, and trihydroxyderivatives called leukotrienes (Chapter 13), some of which are present in atherosclerotic lesions [25]. The monohydroxylated leukotrienes 12(S)- and 15(S)-HETEs can be produced by human arterial endothelial cells and can promote monocyte adherence, an important early event in atherogenesis. Although 15(S)-HETE is found at relatively high levels in human atherosclerotic lesions, its role in vivo is not known. Atheromatous tissue has the capacity to synthesize the dihydroxylated leukotrienes LTC<sub>4</sub> and LTB<sub>4</sub> (R. De Caterina, 1988; C. Patrono, 1992).  $LTC_4$  can be made by monocytes, macrophages, and endothelial cells, and  $LTB_4$  is synthesized by activated monocytes. In theory, LTC<sub>4</sub> could promote vasoconstriction, and LTB<sub>4</sub> could contribute to atherosclerosis-related endothelial alterations, such as increased permeability and adhesiveness. Moreover, LTB<sub>4</sub> is also an activator of PPARα, which appears to promote atherogenesis in vivo (C.F. Semenkovich, 2001). Finally, the trihydroxylated derivatives of arachidonic acid, the lipoxins, possess some anti-inflammatory properties, but it has been speculated that their ability to induce monocyte chemotaxis might promote atherogenesis [25].

Another fate of arachidonic acid with potential relevance to atherosclerosis is cytochrome P450 monooxygenase-derived metabolism to epoxyeicosatrienoic acids (EETs), which may also be formed nonenzymatically by the interaction of arachidonic acid with free radicals [25]. EET synthesis in cultured endothelial cells can be induced by LDL, and EETs are found both in LDL and in human atherosclerotic lesions. Biological effects of EETs include potentially anti-atherogenic effects, such as vasodilatation and prevention of platelet aggregation, and atherogenic responses, such as increased monocyte adhesion.

### 5.4. Atherogenic and anti-atherogenic effects of other long-chain polyunsaturated acids

Dietary intake of n-6 fatty acids such as linoleic acid, and n-3 fatty acids, such as the fish oils eicosapentanoic acid and docosahexaenoic acid, lower plasma cholesterol and antagonize platelet activation, but the fish oils are much more potent in this regard [26]. In particular, n-3 fatty acids competitively inhibit thromboxane synthesis in platelets but not prostacyclin synthesis in endothelial cells. These fatty acids have also been shown to have other potentially anti-atherogenic effects, such as inhibition of monocyte cytokine

synthesis, smooth muscle cell proliferation, and monocyte adhesion to endothelial cells. While dietary intake of n-3 fatty acid-rich fish oils appears to be atheroprotective, human and animal dietary studies with the n-6 fatty acid linoleic acid have yielded conflicting results in terms of effects on both plasma lipoproteins and atherosclerosis. Indeed, excess amounts of both n-3 and n-6 fatty acids may actually promote oxidation, inflammation, and possibly atherogenesis (M. Toberek, 1998). In this context, enzymatic and non-enzymatic oxidation of linoleic acid in the sn-2 position of LDL phospholipids to 9- and 13-hydroxy derivatives is a key event in LDL oxidation (Section 6.2).

### 6. Phospholipids and related lipids

### 6.1. Introduction

Phospholipids compose the outer monolayer of lesional lipoproteins and the membranes of lesional cells. In lipoproteins, the phospholipid monolayer provides an amphipathic interface between the neutral lipid core and the aqueous external environment, and it provides the structural foundation for the various apolipoproteins (Chapter 18). In the specific context of atherosclerosis, the phospholipids of lesional lipoproteins are modified by various oxidative reactions that could have important pathological consequences. In lesional cells, membrane phospholipids not only play structural roles but also are precursors to important phospholipiase-generated signaling molecules that may play important roles in atherogenesis.

### 6.2. Oxidative modification of phosphatidylcholine in lesional lipoproteins

Oxidation of LDL and probably other lesional lipoproteins occurs in atherosclerotic lesions and may contribute to lesion pathology at various stages of atherogenesis [10,11]. Based on in-vitro studies and, in some cases, genetically altered mutant mouse models, LDL oxidation may be triggered by oxidative enzymes secreted by lesional cells, including myeloperoxidase, inducible nitric oxide synthase, and, 15-lipoxygenase. In the above sections, oxidative modifications of cholesterol, cholesteryl ester, and free fatty acids in LDL were discussed. The phospholipids of LDL also undergo oxidative modification, and the products of these reactions are found in atherosclerotic lesions and have potentially important atherogenic effects on lesional cells.

The most abundant and important oxidative changes in LDL phospholipids are those that occur to the unsaturated fatty acids in the *sn*-2 position. Witztum and Berliner described several products of phospholipid oxidation that result from this process [27] (Fig. 7). An early event is the addition of oxygens to these fatty acids, resulting in the generation of hydroxy fatty acids, hydroperoxy fatty acids, and isoprostanes. In one model, lipoxygenase-generated hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE) in LDL surface phospholipids act as 'seeding' molecules. These hydroperoxy fatty acids then trigger the oxidation of arachidonate-containing phospholipids, notably, 1-palmitoy1-2-arachidonyl-*sn*-glycero-3-phosphorylcholine. This early series of events occurs before any oxidative





Phosphorylcholine-CH2



Phosphorylcholine-CH<sub>2</sub>



Phosphorylcholine-CH2

Fig. 7. Oxidation of LDL phospholipids in the generation of minimally modified LDL. 'Seeding' molecules like HPETE, HPODE, and cholesteryl linoleate hydroperoxide (CE-OOH) are proposed to trigger the oxidation of 1-palmitoyl-2-arachadonoyl phosphatidylcholine in LDL. This leads to the generation of three oxidized phosphatidylcholine species that confer atherogenic activity to minimally modified LDL. (Adapted from Navab et al. [28].)

12-LO

PETE, HPODE Chol. Linoleate

modification of apo B100 and result in the generation of so-called 'minimally modified' LDL. Minimally modified LDL is found in atherosclerotic lesions and promotes monocyte binding to endothelial cells and monocyte chemotaxis in cultured cell studies [28]. The oxidized arachidonate-containing phospholipids, which can account for much of the biological activity of minimally modified LDL, include those in which the arachidonyl group is modified to 5-oxovaleroyl, glutaroyl, or 5,6-epoxyisoprostane phosphatidylcholine. The potentially atherogenic molecules induced in endothelial cells and smooth muscle cells by these oxidized phospholipids include E-selectin, VCAM-1, monocyte chemoattractant protein-1, macrophage colony stimulating factor, P-selectin, and interleukin-8. These cellular effects, like those of  $LTB_4$  (Section 5.3), likely involve the activation of PPAR $\alpha$ .

Another possible consequence of oxidation of sn-2 unsaturated fatty acids in oxidized LDL is fragmentation of the fatty acid, resulting in a phospholipid with a short acyl group in the sn-2 position [27]. If the sn-2 fatty acid were arachidonate, the truncated acyl group would be a 5-carbon aldehyde or a 5-carbon carboxylic acid. If the sn-2 group were linoleate, the products would be a 9-carbon aldehyde or carboxylic acid. Phospholipids containing these shortened *sn*-2 acyl chains have biological activity similar to that of platelet-activating factors, where an ether-linked fatty acyl group occupies the sn-1 position and an acetyl group is in the sn-2 position. By interaction with a G-protein-coupled receptor on a variety of cell types, platelet activating factor activates both platelets and leukocytes and increases vascular permeability. In terms of atherosclerosis, studies with PAF receptor antagonists have suggested that the chemotactic activity of minimally modified LDL may be mediated through platelet activating factor-like phospholipids acting directly on monocytes (P.D. Reaven, 1997). Similarly, the smooth muscle cell mitogenic activity of oxidized LDL can be mimicked by PC containing a 5-carbon carboxylic acid and can be blocked by a platelet activating factor receptor antagonist (S.M. Prescott, 1995).

Phospholipase A2-mediated hydrolysis of oxidized phospholipids can result in the release of either intact oxygenated free fatty acids by phospholipase A<sub>2</sub>, discussed in Section 5, or fragmented fatty acids, such as malondialdehyde, which can lead to protein modification. The other product of this reaction is lysophosphatidylcholine (D. Steinberg, 1988) [29]. In-vitro studies have revealed multiple effects of lysophosphatidylcholine on lesional cells, including expression of adhesion molecules on endothelial cells, monocyte chemotaxis and macrophage scavenger receptor expression, growth factor expression by smooth muscle cells and macrophages, cellular cytotoxicity, and inhibition of T cell activation. Although it is not known whether enough lysophosphatidylcholine exists in lesions to cause these effects in vivo, a receptor for lysosphospholipids, called G2A, was recently identified, and studies with G2A-deficient mice suggest a possible role for lysophospholipids in the regulation of T cell activation in vivo (O.N. Witte, 2001). Finally, adducts of oxidized phospholipids and apo B100 can result from LDL oxidation. These adducts are recognized by macrophages and thus can mediate cellular uptake of oxidized LDL, and they form a potent epitope that can elicit cellular and humoral immune responses that may play important roles in atherogenesis (J.L. Witztum, 1999).

Navab et al. have suggested that one of the anti-atherogenic mechanisms of HDL may be its ability to prevent LDL oxidation or reduce its atherogenic activity [28]. Multiple mechanisms may be involved, including removal of the 'seeding' molecules HPODE and HPETE and degradation of the oxidized phospholipids themselves by paraoxonase, an esterase/peroxidase, and platelet-activating factor-acetylhydrolase, a



Fig. 8. Phospholipids whorls in cholesterol loaded macrophages. (A) Electron micrograph showing a membrane whorl in the cytoplasm of a foam cell. The cell was isolated from an advanced aortic atherosclerotic lesion in a cholesterol-fed rabbit. (From Shio et al. (1979) Lab. Invest. 41: 160–167.) (B) Electron micrograph showing a membrane whorl in the cytoplasm of an FC-loaded J774 macrophage. The cell was in the 'adaptive' stage, i.e., before the onset of FC-induced death. (From Shiratori et al. (1994) J. Biol. Chem. 269: 11337–11348.)

lipoprotein-bound phospholipase  $A_2$ -like enzyme that can cleave oxidized acyl groups from the *sn*-2 position of oxidized phospholipids. In apo E knockout mice, targeted disruption of the paraoxonase gene increases lipoprotein oxidation and atherosclerosis (A.J. Lusis, 2000).

### 6.3. The phospholipids of lesional cells

Phosphatidylcholine is the major phospholipid of lesional cells and, as mentioned above, serves both structural and signaling functions. In terms of cellular membrane structure, the cholesterol : phospholipid ratio in lesional cells must be kept within a certain limit in order for the proper functioning of membrane proteins [9]. Cholesterol-rich foam cells isolated from atherosclerotic lesions have intracellular phospholipid whorl-like structures, and PC biosynthesis is increased in lesional areas of the arterial wall (Fig. 8). Cell culture studies have revealed that free cholesterol (FC) loading of macrophages directly leads to the activation of CTP : phosphocholine cytidylyltransferase and an increase in phosphatidylcholine biosynthesis and mass. Proof that this is an adaptive response to FC excess comes from a study in which the cytidylyltransferase $\alpha$  gene was disrupted in macrophages, which resulted in accelerated FC-induced death (I. Tabas, 2000). Thus, activation of phosphatidylcholine biosynthesis in cholesterol-loaded lesional macrophages may help to protect these cells from the toxicity of FC excess [9].

Cellular phospholipids, particularly phosphatidylinositol and phospholipids containing unsaturated fatty acids in the *sn*-2 position, are precursors to a variety of signaling molecules (Chapter 12). These include (1) diacylglycerol and inositol trisphosphate by phosphatidylinositol-specific phospholipase C-induced hydrolysis of phosphatidylinositol, (2) phosphatidic acid by phospholipase D, (3) fatty acids and lysophosphatidylcholine by phospholipase A2, and (4) platelet activating factor-like molecules by oxidation. Diacylglycerol activates protein kinase C, and inositol triphosphate leads to intracellular calcium release. Both of these reactions are involved in a variety of signaling processes that occur in lesional smooth muscle cells, macrophages, and endothelial cells, including responses to cytokines and growth factors. Oxidized LDL has been shown to activate phospholipase D in cultured vascular smooth muscle cells by a tyrosine kinase-mediated mechanism, and phosphatidic acid could mimic the proliferative effects of oxidized LDL in these cells (S. Parthasarathy, 1995). Finally, given the potential importance of apoptosis of macrophages and smooth muscle cells in atherosclerosis (above), phosphatidylserine is an important phospholipid in lesional cells. Phosphatidylserine is normally a component of the inner leaflet of the plasma membrane, but it becomes externalized during apoptosis and acts as a recognition signal and ligand for phagocytes. Interestingly, both CD36 and scavenger receptor B-1 on macrophages are receptors for phosphatidylserine on apoptotic cells as well as for oxidized LDL. Thus, it is possible that phagocytosis and clearance of apoptotic cells in lesions may be competitively inhibited by oxidized LDL (D. Steinberg, 1999).

#### 6.4. Sphingomyelin and ceramide

Sphingomyelin is an important component of both the phospholipid monolayer of lesional lipoproteins and of membranes of lesional cells. In atherogenic lipoproteins like LDL, hydrolysis of sphingomyelin to ceramide results in lipoprotein aggregation and fusion, resulting in the formation of large aggregates that appear similar to those that occur in extracellular regions of the subendothelium of atherosclerotic lesions [8]. The mechanism of sphingomyelinase-induced aggregation and fusion, which is dependent on lipoprotein ceramide content, probably lies in both the physical effects of ceramide on lipoprotein structure and on hydrogen bonding between ceramide on one particle and phospholipids on a neighboring particle. Tabas and coworkers have provided evidence that extracellular hydrolysis of LDL-sphingomyelin by sphingomyelinase occurs in the subendothelium of atherosclerotic lesions and is catalyzed by a form of acid sphingomyelinase, called S-sphingomyelinase, that is secreted by endothelial cells and macrophages [13]. Although the overall importance of this reaction in vivo remains to be determined, its potential importance is that subendothelial lipoprotein retention promotes lipoprotein retention in the arterial wall and is a potent substrate for macrophage and possibly smooth muscle cell form cell formation. Lipoproteins with a high sphingomyelin: phospholipid ratio are particularly good substrates for S-sphingomyelinase. In this context, lipoproteins isolated from atherosclerotic lesions have a very high sphingomyelin (as well as ceramide) content. Moreover, a recent analysis of plasma samples from a case: control study showed that a high sphingomyelin: phospholipid ratio in plasma lipoproteins was an independent risk factor for coronary artery disease in humans (X.C. Jiang, 2000).

HDL also contains sphingomyelin. Because sphingomyelin avidly binds cholesterol, sphingomyelin may increase the ability of HDL to act as an extracellular acceptor for

cholesterol effluxed from cells (G. Rothblat, 1997). However, HDL-sphingomyelin has also been shown to inhibit the binding of lecithin: cholesterol acyltransferase to the lipoprotein, and so this effect may balance the effect of HDL-sphingomyelin-induced cholesterol efflux on reverse cholesterol transport (A. Jonas, 1996).

Sphingomyelin in cellular membranes may have several important roles related to atherogenesis. Because sphingomyelin interacts strongly with cholesterol, accumulation of sphingomyelin in cellular sites that are involved in cholesterol trafficking may influence cellular cholesterol distribution. For example, the defective intracellular trafficking and ACAT-mediated esterification of oxidized LDL-derived cholesterol in macrophages may be due to the inhibition of acid sphingomyelinase by oxidized LDL lipids (M. Aviram, 1995). Moreover, the sphingomyelin accumulation that occurs in acid sphingomyelinase-deficient macrophages leads to defective cholesterol trafficking and efflux (I. Tabas, 2001). On the other side of this issue is the response of sphingomyelin biosynthesis to FC loading of cells. Investigators have shown that the synthesis and mass of cellular sphingomyelin is increased in advanced atherosclerotic lesions [16]. In cultured FC-loaded macrophages, sphingomyelin biosynthesis is increased, suggesting that sphingomyelin, like phosphatidylcholine, plays a role in the adaptation of cells to FC excess.

Another area of sphingomyelin biology with potential relevance to atherosclerosis is related to cell signaling [30]. Hydrolysis of cellular sphingomyelin by either neutral or acid sphingomyelinases results in the generation of intracellular ceramide, which is involved in a variety of cell-signaling reactions (Chapter 14). In terms of atherosclerosis, ceramide-mediated signaling may play roles in smooth muscle cell proliferation and apoptosis and macrophage apoptosis [30]. Alterations in ceramide synthesis and ceramide hydrolysis by cellular ceramidases may also influence these events. In this context, ceramidase-generated sphingosine can be phosphorylated to sphingosine-1-phosphate, which is another signaling molecule. For example, Ross and colleagues showed that sphingosine-1-phosphate blocks the migration of vascular smooth muscle cells induced by platelet-derived growth factor (R. Ross, 1995).

#### 6.5. Glycosphingolipids

Sugar transferases can convert ceramide to a variety of glycosphingolipids, including neutral glycosphingolipids such as glucosylceramide and lactosylceramide, and polar glycosphingolipids such as gangliosides, which contain ceramide, sugars, and sialic acid and/or *N*-glycolylneuraminic acid (Chapter 14). Glycosphingolipids are found both in plasma lipoproteins and in the cells and extracellular regions of atherosclerotic lesions [30]. Chatterjee and colleagues have proposed that lactosylceramide, synthesized from glucosylceramide by the enzyme UDP-galactose: glucosylceramide- $\beta$ 1-4 galactosyltransferase activity (GalT-2), is a lipid second messenger that is involved in the proliferation of vascular smooth muscle cells by oxidized LDL [30]. In cultured smooth muscle cells, oxidized LDL stimulated GalT-2 activity and lactosylceramide synthesis. Proliferation induced by oxidized LDL in these cells was blocked by an inhibitor of GalT-2, and exogenous lactosylceramide was able to stimulate proliferation in the absence of oxidized LDL. The mechanism may involve 5-oxovaleroyl

phosphatidylcholine-mediated stimulation of NADPH oxidase by lactosylceramide, leading to signaling cascade triggered by superoxide radicals and involving Ras activation and p44-mitogen-activated protein kinase. Interestingly, native LDL was shown to actually decrease GaIT-2 activity and lactosylceramide synthesis in smooth muscle cells in an LDL receptor-dependent manner. While these cell-culture studies have provided a potentially interesting role for GaIT-2 activity and lactosylceramide in atherosclerosis, the physiologic significance of these findings overall must await future in-vivo studies.

Lipid	Overall effects in atherosclerosis	Specific examples	
Free cholesterol	Accumulation in and alteration of macrophages and smooth muscle cells, including gene regulation and, in excess, death	Stimulation of ACAT Repress transcription of LDL receptor gene FC-induced macrophage death	
Cholesteryl ester	Accumulation in macrophages and smooth muscle cells Substrate for oxidation	Foam cell formation Cholesterol linoleate hydroperoxide as a pro-oxidant	
Oxysterols	Regulation of cellular cholesterol metabolism Cytotoxicity Sterol efflux pathways Activation of nuclear transcription factors	Stimulation of ACAT 7K-induced macrophage death Efflux of 27OH Activation of LXR by 22OH	
Triglycerides	Source of fatty acids Affect neutral lipid droplet fluidity in foam cells	Liquid crystalline → liquid neutral transformation of foam cell droplets	
Fatty acids	Stimulate CE, triglyceride, and phospholipid synthesis Polyunsaturated fatty acids are sources of bioactive eicosanoids	Thromboxane $A_2 \rightarrow$ platelet aggregation Isoprostanes $\rightarrow$ smooth muscle cell proliferation	
Phospholipids (other than sphingolipids)	Structural roles in lipoproteins and lesional cells Source of signaling molecules Substrate for oxidative modification into bioactive molecules	Part of adaptive response to FC-induced cytotoxicity Lysophosphatidylcholine $\rightarrow$ monocyte chemotaxis Oxidized phospholipids $\rightarrow$ induction of endothelial adhesion molecules	
Sphingolipids	Source of signaling molecules Involve on lipoprotein aggregation Influence intracellular cholesterol trafficking	Ceramide $\rightarrow$ lesional cell death and proliferation Sphingomyelinase-induced LDL aggregation Lactosylceramide $\rightarrow$ smooth muscle cell proliferation	

Table 2 Summary of proposed roles of lesional lipids in atherosclerosis

# 7. Future directions

The potential roles of the many types of lesional and lipoprotein lipids in atherogenesis is staggering (Table 2). Not surprisingly, most studies investigating these roles have been conducted on cultured cells where the concentrations of the lipids and the overall state of cells may be very different from that occurring in atherosclerotic lesions. Thus, one of the most important, and difficult, areas in future studies will be to sort out these effects in vivo through the use of inhibitory compounds or genetic manipulations in mice. In some cases, such studies have provided impressive results, such as the decrease in atherosclerosis observed in 15-lipoxygenase knockout mice (C.D. Funk, 1999). On the other hand, the effects of anti-oxidants in both humans and animal models have vielded conflicting results (R. Stocker, 2001). Further understanding of the molecular basis of lipid synthesis and catabolism, and of the action of bioactive lipids in cells, will help in the design of improved in-vivo models. The most important of these lipids include cholesterol, oxidized phospholipids and fatty acids, oxysterols, and sphingolipid derivatives. Key areas for investigating the cellular effects of bioactive lipids include (1) inflammatory responses in endothelial cells, T cells, and macrophages, (2) secretion of atherogenic and anti-atherogenic molecules by lesional cells, (3) proliferation of macrophages and smooth muscle cells, (4) and apoptotic and necrotic death in lesional cells. Moreover, the mechanism and consequences of macrophage and smooth muscle cell lipid accumulation, particular cholesteryl ester and free cholesterol accumulation, represent fundamental areas in lesional cell biology that require further investigation. New advances in genomics and proteomics have already begun to aid in these effort and will increasingly do so. Ultimately, the goal of these studies is to elucidate novel targets for drug or gene therapy that can complement plasma cholesterol-lowering therapy in the fight against the leading cause of mortality worldwide.

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