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Role of cholesterol and lipid organization in disease

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Membrane lipids are essential for biological functions ranging from membrane trafficking to signal transduction. The composition of lipid membranes influences their organization and properties, so it is not surprising that disorders in lipid metabolism and transport have a role in human disease. Significant recent progress has enhanced our understanding of the molecular and cellular basis of lipid-associated disorders such as Tangier disease, Niemann–Pick disease type C and atherosclerosis. These insights have also led to improved understanding of normal physiology.

The lipid components of biological membranes are important for normal cell function, and their improper distribution or metabolism can have serious consequences for cells and organisms. Some of the important functions of membranes — such as providing a permeability barrier that separates compartments in eukaryotic cells — have been appreciated since the first observations of subcellular organelles. Other functions, such as signalling by phosphoinositides, have also been studied for decades, but recent advances indicate new ways in which these signalling mechanisms can be regulated both spatially and temporally. In the past few years, several lines of evidence have shown that the biophysical properties of membrane bilayers have significant effects on the properties of membrane proteins[1].

Changes in the organization of lipids can have profound effects on cellular functions such as signal transduction and membrane trafficking[2–4]. These membrane effects can cause disease in humans as a result of genetic alterations or environmental effects (such as diet), or both. Cholesterol is one of the most important regulators of lipid organization, and mammals have developed sophisticated and complex mechanisms to maintain cellular cholesterol levels in membranes within a narrow range[5]. When these homeostatic mechanisms are overwhelmed, as in the late stages of atherosclerosis, the consequences can be severe.

Our understanding of the contributions of membranes to disease varies from disorder to disorder. The role of cholesterol and lipids in atherosclerosis has been studied for decades[6], and many of the cellular and molecular mechanisms have been worked out in considerable detail. This has been one of the leading examples (perhaps the best example) of how modern tools of cell and molecular biology can result in understanding and treatment of human disease. However, even in this case, there are important unresolved questions about how cholesterol affects cells in atherosclerotic lesions, how cholesterol moves within cells and how cholesterol is exported to extracellular acceptors.

With other disorders, such as the inherited lysosomal storage diseases (which lead to lipid accumulation in cells), the molecular defects have been identified, but it is often not clear how these defects lead to the particular set of symptoms that afflict patients or how to relieve these symptoms. In other cases, there are tantalizing hints that membrane organization is important, but the details remain very uncertain. For example, polymorphisms in the apolipoprotein Apo-E are strongly linked with the age of onset of Alzheimer's disease, but the basis for this linkage remains unclear. Similarly, treatment with cholesterol-lowering statins has been reported to have beneficial effects in delaying the average onset of Alzheimer's disease, but the cellular and molecular basis for these effects are not clear[7].

In this review, we will briefly summarize the current state of knowledge of membrane organization and lipid trafficking in mammalian cells. We then discuss how changes in lipid composition and organization can lead to altered cell function, and, where possible, we will relate this to our understanding of the pathophysiology associated with these disorders.

Membrane organization

The membranes of mammalian cells have several functional roles that must be carried out simultaneously. The membranes provide a permeability barrier that allows different ion and solute concentrations to exist on each side of the membrane. This allows specialized functions in various organelles and maintains transmembrane electrical potentials. At the same time, membranes provide a scaffold for supporting membrane proteins and yet they must be fluid enough to allow rapid diffusion of these proteins. Membranes must also be flexible enough to bend, for example, when budding to form vesicles or tubules, or fusing with other membranes during trafficking. It is now understood that certain lipids, especially the phosphoinositides, are used by cells to organize signal-transduction processes at certain locations within cells. Furthermore, many biological membranes have a lateral inhomogeneity (microdomains) that can be used to help bring signalling molecules (both lipids and membrane proteins) together or to keep them apart under various conditions[2–4].

The competing demands of these functions place stringent restrictions on the lipid compositions of membranes. For example, highly ordered membranes can generally provide a better permeability barrier than more disordered membranes because polar molecules can more easily intercalate into the disordered lipids. However, highly ordered (gel-phase) lipids would not allow rapid diffusion of membrane proteins, and they would be difficult to bend into vesicles and tubules. In fact, gel-phase lipids are not observed in mammalian cell

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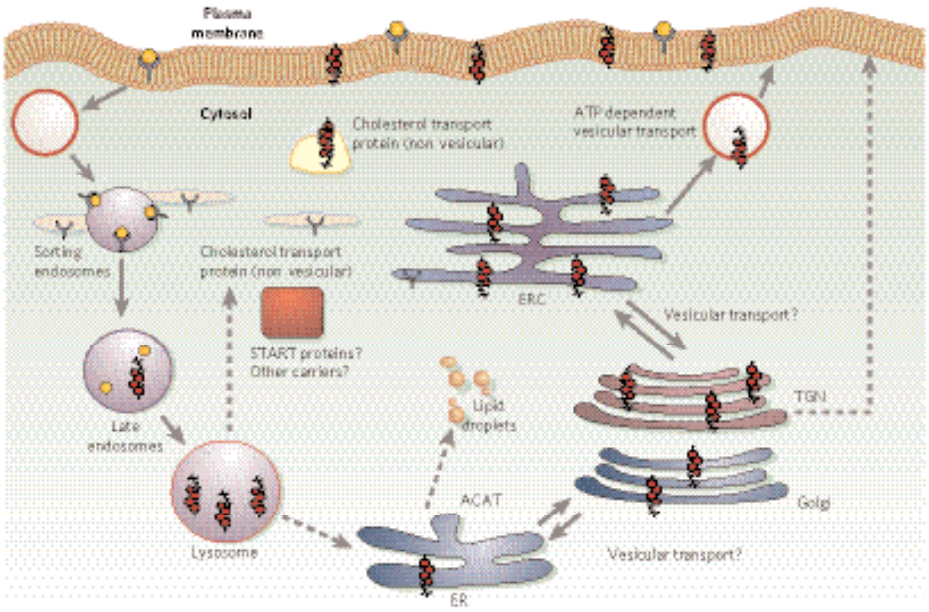


Figure 1 | Intracellular cholesterol transport. LDL (yellow circles) carrying cholesterol and cholesterol esters bound to LDL receptors (light blue Y-shape) is internalized and transported to sorting endosomes and to late endosomes and lysosomes from which cholesterol can efflux to cellular compartments including the plasma membrane or the endoplasmic reticulum (ER). The LDL receptor recycles to the plamsa membrane via the endocytic recycling compartment (ERC). Efflux from late endosomes and lysosomes is poorly characterized as indicated by the dashed lines. Cholesterol can move from the plasma membrane to the ERC by a non-vesicular, ATP-independent process. Recycling of cholesterol back to the plasma membrane occurs by non-vesicular transport and in membrane-recycling vesicles carrying other recycling membrane components. Newly synthesized cholesterol in the ER is mostly transported from the ER directly to the plasma membrane, bypassing the Golgi, but some follows the biosynthetic secretory pathway from the ER to the Golgi. Excess cholesterol in the ER becomes esterified by ACAT and stored in cytoplasmic lipid droplets. TGN, trans-Golgi network.

membranes, which exhibit more dynamic liquid-phase properties. In biological membranes, there is often a mixture of liquid-disordered (l_d) and liquid-ordered (l_o) phases (see Box 1) with the abundance of these types dependent upon lipid composition.

Two or more lipid phases can coexist within a single bilayer, and the importance of this in biological membranes has been studied intensely in the past few years[2,3,8]. The plasma membrane has been studied most, and there is considerable evidence that small domains (microdomains) coexist with different lipid organizations. At present, however, there is considerable uncertainty about the abundance, size, duration and composition differences of the different types of microdomain, and there remain some unanswered questions about whether they exist at all[9]. On the basis of lipid composition, sensitivity to detergent extraction and biophysical measurements of the motion of lipid analogues, it seems that a large fraction, and perhaps the majority, of the lipids in the plasma membrane are in an l_o type of organization[3].

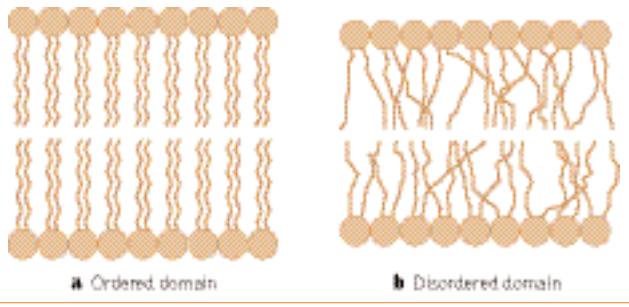
The different types of lipid organization have important effects on membrane proteins. First, many membrane proteins prefer to associate with a particular type of organization, and this will lead to their physical separation in bilayers with coexisting lipid phases[8,10]. For proteins with lipid or fatty-acid anchors, this preference will be determined largely by the properties of the acyl chains. For example, glycosylphosphatidylinositol (GPI)-anchored proteins have a preference for ordered domains because their phosphoinositide anchors typically have saturated acyl chains[11]. Similarly, many palmitoylated and myristoylated proteins (for example, Src-family kinases) associate with ordered domains in the cytoplasmic leaflet[12]. Prenylated membrane anchors (for example, on Ras superfamily GTPases) have a preference for disordered domains because of the unsaturation of the isoprenyl groups[13,14]. It should be pointed out, however, that these preferences on the basis of hydrophobic membrane anchors may be outweighed by interactions with other proteins or by headgroup interactions.

Transmembrane proteins also have preferences for l_o or l_d membranes[8,10]. Most transmembrane proteins seem to have a preference for l_d domains, according to evidence of their sensitivity to mild detergent extraction. Physically this may be related to the difficulty of accommodating a protein transmembrane domain within a tightly packed lipid bilayer without disturbing the lipid organization. Nevertheless, some transmembrane proteins show a preference for more ordered domains, and this is often more pronounced when the proteins are crosslinked[4]. This organizational preference is important in signal-transduction processes and in sorting during membrane trafficking. More ordered membranes have thicker lipid bilayers (see Fig. 1), and this can lead to preferential inclusion of membrane proteins with longer hydrophobic sequences in their transmembrane domain. Organizational preferences have been proposed to influence the sorting of proteins in the secretory pathway[10,15], although this view has recently been challenged by measurements of average bilayer thickness in isolated organelles[16].

One of the most important proposed roles for membrane domains is in the regulation of signal transduction. In particular, crosslinking some signalling receptors can lead to formation of signalling complexes that are associated with ordered, raft-like membranes[4,8]. One of the best-characterized examples is the crosslinking of IgE receptors, which are found on mast cells and are involved in triggering allergic reactions. Crosslinking of these receptors increases their resistance to solubilization by cold Triton X-100, indicating that they are recruited to more ordered membrane domains. Another signalling protein

Box 1 | Ordered and disordered lipids

In ordered lipid phases, the atoms of the acyl chains are tightly packed and relatively elongated (Box 1 Fig. 1a). The disordered lipid phases (l_d) are characterized by rapid diffusion in the plane of the membrane and a poorly ordered structure in the hydrophobic core of the bilayer (Box 1 Fig. 1b). Lipids with unsaturated fatty acids, which have kinks in their acyl chains, increase the propensity of a bilayer to be in an ld organization. The atoms in this phase are not tightly packed, which allows water molecules and other small molecules to penetrate into the bilayer relatively easily. For similar reasons, this type of lipid organization is very susceptible to solubilization by mild detergents[92]. The thickness of the bilayer also decreases as the acyl chains of the lipids become disordered.



recruited to these ordered domains is the Src-family kinase Lyn. Recent studies indicate that Lyn in these ordered domains is protected from an inactivating transmembrane phosphatase, and this leads to greater net phosphorylation of the IgE receptor[4]. Depletion of cellular cholesterol prevents the recruitment of Lyn and the IgE receptor to detergent-resistant ordered domains and abrogates signalling. Placing the cytosolic domain of protein tyrosine phosphatase α on a myristoyl and palmitoyl anchor, which allows it to enter ordered membrane domains, prevented phosphorylation of crosslinked IgE receptors[4]. These results suggest that lipid microdomains are important for segregating proteins on the basis of their preferences for different types of lipid order. This is one way that signalling can be regulated.

In many other cases, the mechanisms by which lipid ordering affects signalling is less clear. Cholesterol is an important determinant of membrane organization (Box 2), and it is relatively easy to manipulate cellular cholesterol levels in cell culture. Thus, cholesterol depletion is often used as a method to test whether lipid organization plays a part in a signalling pathway. A limitation of this is that the levels of cholesterol depletion are often non-physiological, and nonspecific effects can be caused by severe cholesterol depletion. Nevertheless, cholesterol depletion, along with other data such as detergent resistance, can indicate a role for lipid organization in signalling. Several studies have suggested that there is a relationship between lipid domains and the actin cytoskeleton[4]. Moderate reductions in cholesterol cause an inhibition of neutrophil motility and a marked reduction in actin-dependent protrusions on human neutrophils in response to chemoattractants, and similar results are seen in other cell types[17]. Some of these effects are associated with reduced activation of the small GTPase Rac in cells, with an approximately 30% reduction in cholesterol, but the precise mechanism linking lipid organization to Rac signalling is not known.

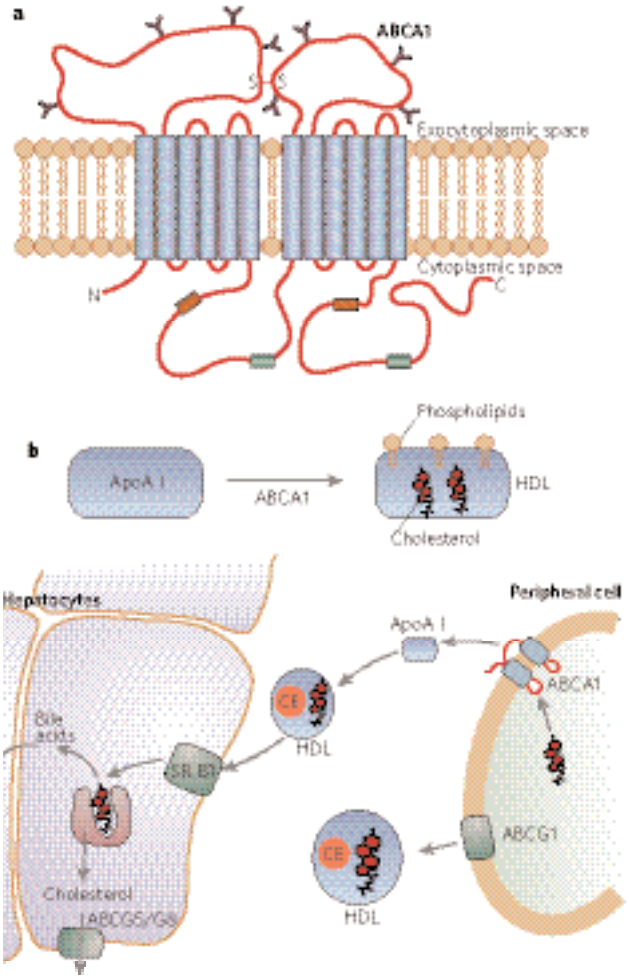


Figure 2 | Cholesterol efflux. a, ABCA1 is a multiple membrane-spanning protein with two nucleotide-binding folds linked by a cytoplasmic peptide sequence. **b**, ABCA1 promotes the transfer of phospholipids to lipid-poor forms of ApoA-I, the major protein component of HDLs. The mechanisms for this transfer are not fully understood, but ABCA1 apparently functions by translocating phospholipids and perhaps cholesterol across the plasma membrane bilayer and presenting them to ApoA-I, which binds to ABCA1 (refs 39, 43). **c**,These ApoA-I particles are transformed into HDLs in the blood by the action of lysolecithin:cholesterol acyltransferase (LCAT). ABCG1 promotes the efflux of cholesterol to larger HDL particles[99,100]. The HDLs bind to scavenger receptor-B1 (SR-B1) in hepatocytes, and transfer their associated cholesterol and cholesterol esters to the liver. The cholesterol is excreted into the bile either as free cholesterol (by way of ABCG5/G8) or after conversion to bile salts[43].

Membrane bilayer properties can affect the activity of single proteins — particularly proteins with multiple membrane-spanning domains that undergo conformational changes as part of their activity cycle[1]. For example, the Ca^{2+}-ATPase from the sarcoplasmic reticulum, which pumps Ca^{2+} into the sarcoplasmic reticulum of muscle cells, can be reconstituted into vesicles made up of phospholipids with various fatty-acid lengths. The ATPase activity is greatest when the lipids approximately match the bilayer thickness of cellular membranes. The changes in bilayer thickness would be expected to affect the differences in free energy of the different conformational states of the protein as it goes through its activity cycle, and this could affect the kinetics of the pump activity. The activity of the Na^+,K^+-ATPase reconstituted into artificial membranes can be altered by the addition of cholesterol to the membranes, but it is uncertain whether this is due to changes in biophysical properties of the bilayer or to specific associations with cholesterol[1]. For several proteins, a specific sterol-sensing domain has been identified. In the sterol-regulatory-element-binding protein (SREBP) cleavage-activating protein (SCAP), there is a particular sequence in a transmembrane domain that is responsible for binding cholesterol and inducing a conformational change. This allows SCAP and its associated SREBP to be transported out of the endoplasmic reticulum (ER) when cellular cholesterol is low[18]. These results emphasize that lipids can have effects on protein function both through specific binding and through changes in the bilayer biophysical properties.

Different organelles within a cell have distinct lipid compositions. The plasma membrane has a high concentration of cholesterol, whereas the outer leaflet of the plasma membrane has high levels of sphingomyelin and glycosphingolipids. This composition is consistent with a highly ordered membrane. The endocytic recycling compartment[19] and parts of the *trans*-Golgi[8] are also relatively highly ordered membranes. At the opposite extreme, the ER has a low cholesterol content and a large fraction of unsaturated lipids, which contribute to a more disordered membrane organization. It is unclear how this type of lipid organization contributes to the function of the ER. One possibility is that it facilitates activities such as the flipping of dolichol-linked glycoconjugates and certain lipid precursors from the cytosolic leaflet to the luminal side of the ER membrane.

Cholesterol is synthesized in the ER and delivered to other organelles by a combination of vesicular and non-vesicular transport processes[20–22] (Fig. 1). The mechanisms of non-vesicular cholesterol transport are only partly understood, but there is substantial evidence that this is the major route for cholesterol movement between organelles. Because cholesterol is very insoluble in water, it must be shuttled by carriers. A few candidates for cholesterol carriers in the cytoplasm have been identified[22], but in most cases proof of their role is still lacking. Perhaps the best-documented example is the steroidogenic acute regulatory protein (StAR), which is the prototype for the StAR-related lipid transfer (START) family of transport proteins. StAR plays an essential role in the delivery of cholesterol to mitochondria, where it is used in steroid hormone synthesis in steroidogenic tissues. Other members of this family of proteins can bind cholesterol or other lipids and facilitate their intracellular, non-vesicular transport[23]. Stud-

ies of the transport of a naturally fluorescent sterol, dehydroergosterol (DHE), have shown that sterols can have a very high flux through the cytoplasm[19,24]. For example, in Chinese hamster ovary cells, in which about 40% of the DHE is in the endocytic recycling compartment, the DHE in that compartment can be replenished with a $t_{1/2}$ of 2–3 minutes after photobleaching[19].

The other major source of cellular cholesterol is endocytic uptake of lipoproteins such as low-density lipoprotein (LDL) and hydrolysis of their cholesterol esters in late endosomes and lysosomes[5]. The lipoprotein-derived cholesterol is rapidly released from these hydrolytic organelles and delivered throughout the cell. Studies of Niemann–Pick disease type C (NPC), an inherited lysosomal storage disorder that leads to accumulation of cholesterol and other lipids, have shown that a luminal protein (NPC2; ref. 25) and a transmembrane protein (NPC1; ref.26) in late endosomes are required for efflux of cholesterol from these organelles, but the details of how these proteins work remain to be determined[25,27,28]. In normal cells, these efflux mechanisms keep the cholesterol content in late endosome membranes low.

Homeostatic mechanisms

Organisms must maintain the proper functioning of their membranes in response to various changes. In humans, one of the most significant factors affecting the membrane is dietary intake of cholesterol and fats, which are delivered to cells throughout the body through lipoproteins[5]. Because cholesterol is a major regulator of lipid organization, its cellular concentration must be maintained within a narrow range, and cells have a variety of mechanisms for accomplishing this. Rapid transport of sterol by vesicular and non-vesicular pathways ensures that any changes are rapidly reflected in changes in cholesterol levels in many organelles.

A rapid response to increasing cholesterol levels is the esterification of excess cholesterol by an ER enzyme, acyl CoA:cholesterol acyltransferase (ACAT)[29]. The esterified cholesterol is stored in cytoplasmic lipid droplets. The cholesterol esters in the droplets are hydrolysed by neutral cholesterol ester hydrolases, which in some cells include a hormone-sensitive lipase that also hydrolyses triglycerides in fat cells[30]. The cholesterol released from the droplets can be used for cell membranes and, in steroidogenic cells, for steroid hormone synthesis. The cycle of cholesterol esterification and hydrolysis may provide the major short-term buffering of cholesterol levels in cells. The activity of ACAT is regulated by cholesterol levels[31], providing a homeostatic sensor. This regulation occurs at two levels: ACAT is allosterically regulated by cholesterol[32], and cholesterol loading of cells promotes more rapid efflux of sterol from the plasma membrane[24], which could increase the rate of delivery to ACAT in the ER[33].

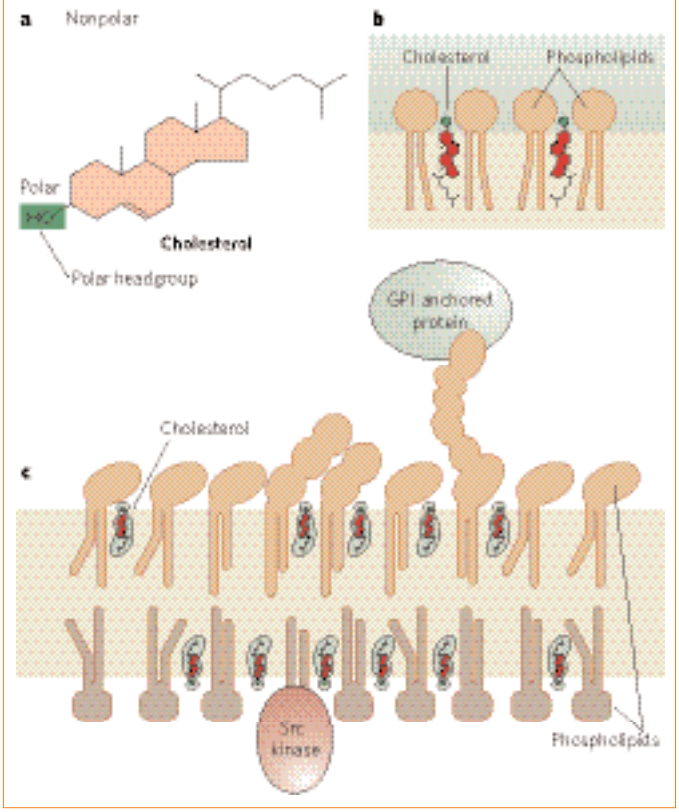
As mentioned earlier, many genes involved in cholesterol metabolism are regulated by SREBP[34]. When cholesterol levels are high, SREBP and SCAP are retained in the ER by binding to INSIG, a resident ER protein. When cholesterol is low, the SREBP–SCAP complex exits from the ER, and SREBP undergoes two proteolytic cleavages. This releases the cytosolic domain of SREBP, which is then translocated into the nucleus and regulates the transcription of many genes, including the LDL receptor and HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. Thus, this system regulates both the synthesis of cholesterol and its uptake through lipoproteins.

A third level of cholesterol regulation within cells is provided by cholesterol efflux mechanisms (Fig. 2). In the past few years, the molecular mechanisms for cellular export of cholesterol have begun to be understood, but there are still significant gaps in our knowledge concerning how these processes operate and how they are regulated. The key extracellular acceptors for cholesterol are high-densiy lipoproteins (HDLs) and one of their associated apolipoproteins, ApoA-I (ref. 35). The family of ABC transporters has a key role in delivering cholesterol and phospholipids to apolipoproteins, and defects in these transporters lead to several human diseases, as discussed below[36]. Indeed, the identification of the gene responsible for Tangier disease, which leads to HDL deficiency and increased cholesterol ester storage in macrophages, was the key to discovering the role of the ABC transporters in cholesterol efflux. The lipid-bilayer properties influence cholesterol efflux, but the precise mechanisms for this are unclear.

In several studies, cholesterol efflux has been linked to expression of caveolin, the coat protein of caveolae, and it has been proposed that these cholesterol-rich membrane domains may be a site of cholesterol

Box 2 | Effects of cholesterol on lipid organization.

The structure of cholesterol is very different from that of other membrane lipids (Box 2 Fig. 1a). The body of cholesterol consists of a series of fused rings, which make that part of the molecule quite rigid. At one end of this planar ring system is a hydroxyl group and at the other end is a hydrocarbon tail, so cholesterol, like other membrane lipids, has both hydrophilic and hydrophobic poles that determine its positioning within the lipid bilayer. When the hydroxyl group is next to the phospholipid ester carbonyl, the rigid body of cholesterol is situated alongside the fatty-acid tails of neighbouring phospholipids and can help to order these tails. Cholesterol can have preferential interactions with certain lipids, either because its small headgroup requires additional shielding from adjacent lipids[93] or through hydrogen-bonded complexes with lipids such as sphingomyelin[94] (Box 2 Fig. 1b). The polar moiety of cholesterol is much smaller than the polar headgroups of other lipids, so flip-flop between leaflets of the membrane bilayer can occur readily. The transbilayer distribution of cholesterol is not known. Cholesterol can increase the order in liquid membranes through the effects of its rigid ring system and the ability to fill interstitial spaces. A particularly important type of lipid organization is the l_o phase[95] in which the atoms in the hydrophobic core are more tightly packed than in the l_d phase, but the lipid molecules are able to diffuse in the plane of the bilayer almost as rapidly as in the l_d phase. The l_o organization provides a good permeability barrier while allowing movement of membrane constituents. Cholesterol is an important component of l_o phase membranes, and its structure seems to allow it to fill interstitial spaces between lipids (providing tight packing), while still allowing rapid diffusion. Cholesterol may also help to stabilize boundaries between coexisting lipid domains[96]. In model membranes other lipids with small polar head groups (for example, ceramide) may be able to also support the formation of an l_o-type of organization[97,98]. The l_o type of lipid order would be associated with detergent-resistant lipid domains or 'rafts'. The ordering of the acyl chains also causes thickening of the bilayer.



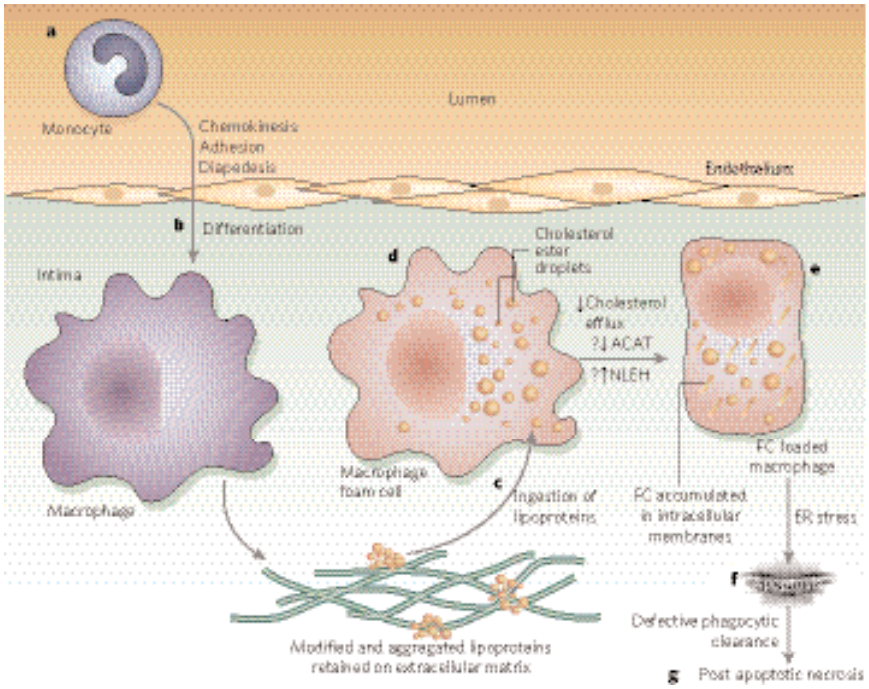


Figure 3 | Entry and cholesterol loading of macrophages in atherosclerotic lesions. **a**, Monocytes are attracted to focal areas of the arterial wall in which atherogenic lipoproteins have been retained on the extracellular matrix. These retained lipoproteins, particularly those whose phospholipids are modified by oxidation, signal to the endothelium to express chemokines and adhesion molecules. **b**, The monocytes then migrate through the endothelial layer and differentiate into macrophages. **c**, The macrophages ingest the retained lipoproteins by endocytic and phagocytic mechanisms and thus acquire a large load of lipoprotein-derived cholesterol. **d**, In early lesions, the cholesterol is stored as ACAT-derived cholesteryl esters and thus acquire a foamy appearance. **e**, In advanced lesions, unesterified or 'free' cholesterol (FC) accumulates, leading to macrophage apoptosis (**f**) and necrosis (**g**).

efflux[37]. How this works is unclear. In cultured cells, overexpression of stearoyl CoA desaturase leads to an increase in unsaturated acyl chains in membrane lipids and a decrease in the resistance of the plasma-membrane lipids to extraction with cold Triton X-100 (refs 38, 39). These changes in bilayer properties are associated with a decrease in cholesterol efflux to apo A-I but an increase in passive release of cholesterol to an HDL2 acceptor[38,39].

In most peripheral tissues, metabolism of cholesterol (other than esterification/de-esterification) is a minor biochemical pathway. However, most cells convert a small fraction of cholesterol into oxysterols[40], and these molecules are important for intracellular signalling. For example, cholesterol overload in cells, which activates the nuclear liver X receptor/retinoid X receptors (LXR/RXR), perhaps through oxysterol intermediates, triggers a 'reverse cholesterol transport' programme involving both cellular cholesterol efflux and transport of the effluxed cholesterol to the liver for secretion in bile[41]. Specifically, activated LXR/RXR leads to the induction of the efflux receptors ABCA1 and ABCG1, the efflux enhancer apolipoprotein E, the plasma lipid transfer proteins CETP and PLTP, stearoyl CoA desaturase, the bile synthetic enzyme Cyp7a and the cholesterol-to-bile transporter ABCG5/G8 (refs 35, 42, 43). In the liver, cholesterol is excreted into the bile both as free cholesterol and after conversion to bile acids.

In addition to regulation of membrane properties by changes in cholesterol, the degree of unsaturation of the acyl chains in phospholipids is an important determinant of membrane biophysical properties. The availability of fatty acids for incorporation into phospholipids is controlled by several factors, including their extracellular availability (for example, through dietary sources), and by a complex network of metabolic regulatory mechanisms. The SREBP family of transcriptional regulators has significant effects on levels of proteins involved in fatty-acid synthesis and modification in addition to their effects on cholesterol synthesis[44]. Furthermore, SREBP as well as other transcriptional regulators of fatty-acid metabolism such as the LXRs, the peroxisome proliferator-activated receptors (PPARs) and hepatocyte nuclear factor can all be regulated by polyunsaturated fatty acids[44,45]. This regulation affects the amount of fatty acids in the cell and the degree of their unsaturation, which can alter the saturation of the fatty acids incorporated into phospholipids as well as the cholesterol:phospholipid ratio in the cell. This regulation can help to maintain the proper biophysical properties of the cell membranes, but it is uncertain whether membrane bilayer properties themselves directly regulate these biosynthetic pathways.

Disorders of lipid and cholesterol metabolism

Although alterations in lipid metabolism and distribution may contribute to many diseases, there are several genetic diseases for which alterations in lipid traffic or metabolism are the primary cause. The study of these disorders has contributed enormously to our understanding of basic mechanisms of lipid metabolism and transport. At the same time, the detailed aetiology of these diseases is often difficult to explain.

Under normal conditions, membrane components that are delivered to late endosomes and lysosomes are subject to hydrolysis by the hydrolytic enzymes in these organelles. This catabolic process is important for the normal turnover of lipid components, and a lack of activity from one of these hydrolases leads to an accumulation of the undegraded substrate for the missing hydrolase. Several lysosomal storage disorders (including Tay–Sachs, Fabry, Niemann–Pick type A or B, and Sandhoff diseases) arise from defects in the breakdown of lipids in late endosomes and lysosomes. These can be caused by defects in a single hydrolytic enzyme or in activator proteins that participate in the digestion of sphingolipids[46]. Normally, the sphingolipids and glycosphingolipids become segregated into internal membranes in late endosomes along with an unusual negatively charged lipid, bis(monoacylglycero)phosphate (BMP), which is also called lysobisphosphatidic acid (LBPA)[47]. These internal membranes can be continuous with the limiting membrane or detached to form internal vesicles. In either case, they appear as 'multi-vesicular bodies' in electron micrographs. When hydrolysis of sphingolipids is impaired, the internal membranes containing sphingolipids and BMP accumulate, and almost the entire lumen of the organelles can become filled with these membranes, which can form a series of internal membrane whorls[46].

NPC disease is a lysosomal storage disorder that shares many characteristics with the lysosomal sphingolipid enzyme deficiencies. It is caused by a primary defect in cholesterol or lipid trafficking rather than an enzymatic deficiency[28,48]. Late endosomes lacking functional forms of either the NPC1 protein or the NPC2 protein show very slow efflux of cholesterol from late endosomes. Because NPC2 is a late endosome luminal protein that binds cholesterol[49], it is likely that cholesterol transport is the primary defect in cells carrying this mutation. For the NPC1 protein, which is a multi-spanning membrane protein,

the mechanism by which it affects cholesterol transport is not known. Internal membranes accumulate in the late endosomes of NPC cells. Additionally, there are changes in the trafficking of various lipid molecules and cholesterol that are similar in NPC and several of the lipid hydrolysis enzyme deficiencies[50]. In effect, these storage organelles become a sink for lipids and cholesterol in the cell. Changes in lipid composition or cholesterol content can alter endocytic sorting of lipids[3], but it is not known precisely how such changes exert their effects. It is plausible that a buildup of one membrane lipid component traps certain other lipids in the internal membrane whorls of the storage organelles. Interestingly, overexpression of Rab7 or Rab9, small GTPases that regulate vesicle trafficking, can partly correct the NPC phenotype of cholesterol storage in tissue culture fibroblasts[50].

In most lysosomal storage diseases, the accumulation of lipids can be seen in many tissues, and it can be observed in cultured fibroblast lines from the affected individuals. Typically, the most serious effects are seen in the brain, which leads eventually to neuronal death and neurological complications that are often severe and frequently fatal. In some cases, the defects appear at early developmental stages. It is unclear precisely how the various types of lysosomal storage disorder lead to cell death. Perhaps dysfunctional catabolism by lysosomes leads to shortages of certain metabolites. Another possibility is that blockage of vesicle transport along microtubules is blocked by the enlarged storage organelles; or maybe levels of important signalling molecules (for example, oxysterols) are reduced because their precursors are not released into the cytoplasm. It is unclear at present if any of these are the important causes of pathology or whether cell death arises by some other mechanism.

Defects in members of the ABC family of transporters are associated with a variety of human diseases. Tangier disease is a very rare autosomal recessive disorder caused by defects in ABCA1 (ref. 51). This is associated with a severe deficiency in HDL and reduced efflux of cholesterol, especially from macrophages and other reticuloendothelial cells. This leads to cholesterol ester accumulation in these cells and is also associated with increased susceptibility to atherosclerosis. ABCG5 and ABCG8 are expressed in the liver and intestines[51]. These proteins can transport cholesterol and other sterols into the bile or the intestines. In the intestines ABCG5 and ABCG8 excrete newly absorbed plant sterols to a much greater extent than cholesterol.

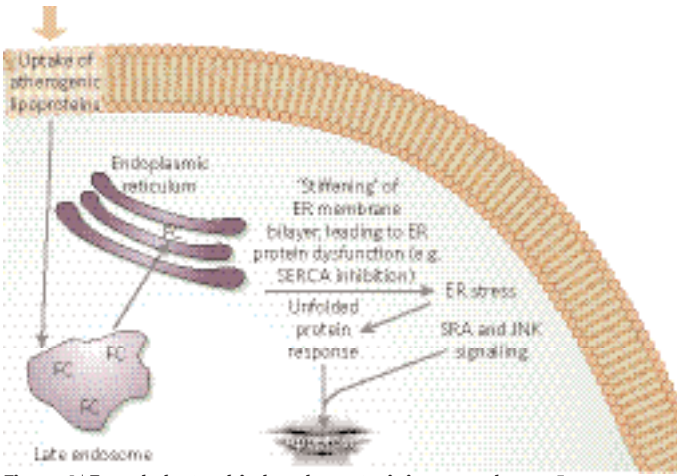


Figure 4 | Free cholesterol-induced apoptosis in macrophages. In macrophages in advanced atherosclerotic lesions, the internalization of atherogenic lipoproteins leads to FC accumulation, perhaps due to defective ACAT and/or overactive neutral CE hydrolase. When the FC:phospholipid ratio in the ER membrane reaches a certain level, integral ER membrane proteins such as sarco(endo)plasmic reticulum ATPase (SERCA) become inactive. Inactivation of SERCA corresponds closely to an increase in order parameter ('stiffening') of the bilayer. Perturbation of ER function by this mechanism leads to activation of the unfolded protein response and other ER stress pathways, which, together with signalling involving the type A scavenger receptor, triggers apoptosis.

Defects in these proteins lead to a rare autosomal recessive disorder, sitosterolaemia, that is associated with a large increase in plasma levels of plant sterols but only modest increases in plasma cholesterol[51]. The buildup in plant sterols, such as sitosterol, is associated with tendon and tuberous xanthomas as well as arthritis and atherosclerosis.

Early events in atherosclerosis

Atherosclerosis is the major human disease associated with cholesterol and lipid metabolism. The earliest detectable event in atherogenesis (the process of forming atheromas) is the accumulation of plasma lipoproteins in the subendothelium, or intima, of focal areas of the arterial tree[52]. The lipoproteins are retained owing to a combination of proteoglycan binding and lipoprotein aggregation, which impedes egress from the arterial wall because of their increased particle size. These retained lipoproteins, particularly those that are modified by oxidation, aggregation and other means, elicit a series of biological responses that lead to atherogenesis[52]. Chief among these biological responses is an unusual type of inflammation consisting of infiltration of monocytes and T cells but not neutrophils[53] (Fig. 3). More specifically, certain types of oxidized phospholipid derived from modified lipoproteins can activate the overlying endothelium to secrete chemokines and express adhesion molecules for monocytes and T cells[54]. These leukocytes migrate through an otherwise intact endothelial layer, and the monocytes eventually differentiate into macrophages in the intima[55].

Once embedded in the intima, the macrophages encounter native and modified lipoproteins, most of which are bound to the matrix. Through a process that is only partly understood but has aspects related to receptor-mediated endocytosis and phagocytosis, the macrophages ingest the lipoprotein particles[56–59]. In a cell-culture model of the initial interaction of macrophages with retained and aggregated lipoproteins, significant rearrangement of the actin cytoskeleton and protrusion of membrane processes is seen, and this is required for the continued uptake of cholesterol into the cells[59]. Interestingly, just as cholesterol depletion inhibits signal-dependent actin assembly in some cells[17], loading of macrophages with cholesterol through uptake of modified lipoproteins or through a cyclodextrin carrier (that is, without a lipoprotein) can lead to increased actin assembly and protrusion of membrane processes in macrophages[60]. It seems likely that these cholesterol-dependent effects are mediated by changes in lipid organization, which can affect activation of the small GTPase Rac[17,60]. In the blood vessel wall, the initial contact with lipoproteins could lead to cholesterol transfer to the macrophages, leading to actin-dependent protrusions. This would enhance the further uptake of cholesterol into the cells.

Most of the cholesterol in lipoproteins is in the form of cholesteryl fatty-acyl esters. These esters are hydrolysed to cholesterol and fatty acids in acidic, degradative organelles such as late endosomes and then transported to other sites in the macrophage. Cholesterol transport to the plasma membrane is important for cholesterol efflux; transport to the ER is necessary for intracellular cholesterol homeostasis (through SREBP) and for re-esterification by ACAT. Transport to the mitochondria leads to the formation of oxysterols, which, in turn, may have roles in LXR activation and sterol efflux. ACAT-mediated re-esterification is a major fate of lipoprotein-derived cholesterol in intimal macrophages. The resulting cholesteryl ester molecules coalesce into membrane-bound neutral lipid droplets in the cytoplasm, a feature that has given rise to the term 'foam cell'[61].

Receptor-mediated uptake by means of the LDL receptor is usually limited because of its homeostatic downregulation by cholesterol. However, aggregated LDL, a major form of LDL in atherosclerotic lesions, can deliver enormous amounts of cholesterol to macrophages and cause foam-cell formation[62]. The likely explanation is that one or more receptors other than LDL receptors are involved and/or that LDL receptor downregulation is not complete in these macrophages. In terms of non-native LDL, a survey of the literature over the past 10–20 years gives the impression that foam cells are formed mostly, if not

exclusively, by the uptake of oxidized LDL. However, most forms of oxidized LDL are not particularly potent inducers of foam-cell formation in cultured macrophages[62]. One explanation for this often-neglected finding is that oxidized LDL-derived cholesterol is poorly trafficked from late endosomes to ACAT in the ER[62]. *In vivo*, there are studies showing the importance of two oxidized LDL receptors — the type A scavenger receptor and CD36 (refs 63, 64) — but these findings have been questioned by a recent study in mice[65]. Moreover, the most reliable anti-oxidant trials in humans have not shown a benefit of vitamin E or other anti-oxidants in decreasing the incidence of atherosclerotic heart disease[66]. Finally, a type of atherogenic lipoprotein often neglected in the discussion of foam-cell formation is the remnant lipoprotein class[67]. Remnant lipoproteins result from the partial catabolism and subsequent cholesterol enrichment of triglyceride-rich lipoproteins made by enterocytes and hepatocytes. Remnant lipoproteins are avidly internalized by cultured macrophages and are potent inducers of ACAT activation and foam-cell formation. Moreover, they are abundant in the intima of atherosclerotic lesions, and their levels in plasma are strongly associated with the presence of foam cells and incidence of atherosclerotic vascular disease in animal models and humans[67]. In summary, a number of 'atherogenic' lipoproteins may cause macrophage foam-cell formation during early atherogenesis, and it is almost certain that a combination of these lipoproteins carry out this role *in vivo*.

Regarding the functions of foam cells in atherogenesis, studies with genetically altered mice have uniformly demonstrated the pro-atherogenic role of macrophage foam cells in early lesions[55]. For example, the lesion area is substantially decreased in mice with defective macrophage development resulting from absent M-CSF or in mice with perturbed monocyte chemokines or chemokine receptors[55]. Similar results are found when early lesional macrophages are depleted by enhanced apoptosis[68]. Although the mechanisms of foam-cell-induced atherogenicity are not known, the ability of foam cells to secrete inflammatory cytokines and matrix metalloproteinases are likely to be contributing factors[55,69]. Macrophage foam cells may also participate in other early atherogenic processes, such as smooth-muscle-cell migration and T-cell-mediated inflammatory and immune responses[69]. A major question is whether these and other roles of foam cells in early atherogenesis are specifically induced by cholesterol loading *per se* or whether cholesterol loading represents a parallel, non-causative event in macrophage-mediated early atherogenesis. Surprisingly, there is a paucity of data addressing this fundamental question.

Late stages of atherosclerosis

Early atherosclerotic lesions are not symptomatic because arterial lumen occlusion is not great enough to compromise blood flow[70]. This lack of occlusion is aided by outward remodelling of the affected region of the arterial wall. After years of gradual lesion development, foam cells, smooth muscle cells, extracellular matrix material and smooth-muscle-cell-derived scar tissue can lead to slowly progressive lumen occlusion, but symptoms are usually absent because organ blood flow is restored by compensatory, hypoxia-driven neovascularization. If this compensatory process becomes compromised, the patient may experience stable, exercise-induced compromise of blood flow (for example, exercise-induced angina) but not acute cardiovascular events[70]. Importantly, the smooth-muscle-cell-derived scar tissue forms a fibrous cap that covers and essentially 'protects' the underlying lesion, and these lesions tend to be relatively stable[71].

A minority of lesions progress to a point in which they precipitate acute vascular events, including sudden death, acute myocardial infarction, unstable angina or ischaemic stroke[71]. These events are caused by acute, occlusive luminal thrombosis, which, because of the suddenness of lumen occlusion, leads to organ damage. This process occurs over minutes, so there is not enough time for compensatory responses. Pathological observations of affected arteries in patients suffering from acute events has led to the plaque-disruption theory of acute atherothrombosis[71–74]. According to this theory, a minority of plaques become necrotic and highly inflammatory, which eventually leads to breakdown of the protective fibrous cap or to erosion of the endothelial cell layer. These events, in turn, expose the luminal blood to underlying plaque material, which promotes coagulation and thrombosis. Of interest, these rare events do not necessarily occur in the largest plaques, but rather those that have large areas of necrosis.

What promotes plaque disruption? According to one theory, late lesional macrophages secrete matrix metalloproteinases, and these enzymes lead to breakdown of the fibrous cap[75]. *In vitro* studies have supported this idea and have suggested that inflammatory mediators promote macrophages to secrete the proteases. However, definitive *in vivo* data for this idea are lacking. Another theory proposes that death of smooth muscle cells promotes plaque instability because intimal smooth muscle cells synthesize the collagen that makes up the protective cap[76]. A third theory holds that macrophage death is important, because it is this event, in the absence of efficient phagocytic clearance of apoptotic cells, that gives rise to the necrotic core[77]. In this regard, there is evidence for defective phagocytic clearance of apoptotic macrophages in advanced atherosclerotic lesions, which leads to post-apoptotic necrosis of the cells[78,79]. By contrast, phagocytic clearance of apoptotic macrophages seems to be intact in early lesions[79]. As mentioned above, there are very strong spatial and temporal correlations between necrotic cores and plaque disruption. Although direct causality has not yet been proven *in vivo*, necrotic cores are rich in proteases, inflammatory molecules and pro-coagulation and thrombosis factors[74,76,79].

There are a number of theories to explain late lesional macrophage death, including exposure to oxysterols, deprivation of growth factors, interaction with cytotoxic cytokines and intracellular accumulation of excess unesterified or 'free' cholesterol (FC)[80,81]. Support for the latter mechanism comes from *in vivo* studies showing that late lesional macrophages accumulate large amounts of FC and from *in vitro* studies showing that FC accumulation is a potent inducer of macrophage apoptosis[31]. It is not known why late lesional macrophages accumulate FC, but it is likely to be due to a combination of perturbed cholesterol esterification and diminished cholesterol efflux. Direct proof for dysfunctional ACAT or efflux proteins (such as ABCA1 and ABCG1) in late lesions is lacking. However, *in vivo* observations are strongly consistent with the idea of a dysfunctional ACAT pathway (below).

Mechanistic studies have begun to reveal a fascinating series of signal-transduction pathways that mediate FC-induced macrophage death[31] (Fig. 4). The key initiating event is trafficking of lipoprotein-derived FC to the ER membrane bilayer, which normally has a low cholesterol:phospholipid ratio and is therefore relatively disordered. Upon enrichment with FC, the order parameter of the ER membrane increases, and this increase is very closely correlated with loss of activity of an integral ER membrane protein, sarco(endo)plasmic reticulum ATPase (SERCA)[82], a protein related to the sarcoplasmic reticulum Ca^{2+}-ATPases found in muscle. The significance of this finding is two-fold. First, it probably indicates that other integral membrane proteins in the ER become dysfunctional in FC-loaded macrophages. Second, loss of SERCA function would be expected to result in depleted ER calcium stores. Indeed, careful measurements have shown that ER calcium pools are depleted within about 2 hours of FC loading, and this event may at least trigger subsequent cellular events[83]. As discussed above, the activity of the SERCA pumps can be affected by changes in bilayer properties, such as thickness, which would be increased upon cholesterol loading.

Within 5 hours of FC loading, upstream and downstream molecules in the ER stress pathway known as the unfolded protein response (UPR) are activated[83]. As alluded to above, one contributing factor could be depletion of ER calcium stores, which renders chaperones dysfunctional and thereby triggers activation of the UPR. However, the dysfunction of other ER membrane proteins might also contribute to UPR activation. One of several UPR effector proteins is a protein called CHOP (GADD153), which can, in turn, affect the transcription

of a number of genes that participate in the UPR programme[84]. In other systems, CHOP can participate in an apoptosis response, and FC-induced apoptosis is markedly inhibited in Chop$^{-/-}$ macrophages[83]. *In vivo* evidence for these events includes the demonstration that apoptotic macrophages surrounding necrotic cores in advanced lesions are filled with FC and show evidence of UPR activation[83,85,86].

Most importantly, macrophages with a heterozygous mutation in the NPC1 have a defect in trafficking of lipoprotein-cholesterol to the ER, and, as expected, this inhibits FC-induced UPR activation and apoptosis despite increased cellular stores of FC[83,85]. Analysis of advanced lesions from *Npc1*$^{+/-}$;*Apoe*$^{-/-}$ mice revealed a marked decrease in late lesional necrosis and macrophage apoptosis compared with similarly sized late lesions of *Apoe*$^{-/-}$ mice[85]. Finally, another consequence of FC-induced ER stress in macrophages is activation of mitogen-activated protein kinase pathways and NFκB[87]. Among the consequences of these events is marked secretion of tumour necrosis factor α and interleukin-6, two inflammatory cytokines that are thought to play important roles in late lesional plaque disruption.

In summary, a series of cell-biological events in a subset of advanced atherosclerotic lesions leads to plaque instability, which, in turn, precipitates acute thrombosis and vascular occlusion. Among these events are secretion of proteases and inflammatory cytokines by macrophages and death of macrophages and smooth muscle cells in the setting of defective phagocytic clearance. Macrophage death seems to be particularly important, because it is this event that gives rise to the destabilizing necrotic core. Increasing evidence suggests that an important cause of late lesional macrophage-mediated inflammation and macrophage death is the accumulation of excess intracellular FC. New studies have revealed a number of signal-transduction pathways, centred on the ER, that account for these cellular effects of FC. Novel therapeutic strategies based on this new insight may provide the means to prevent plaque destabilization and acute atherothrombotic vascular events.

Other diseases

Membrane organization is important for many basic cell functions, and so it would be expected that changes in cholesterol or other aspects of lipid organization have a role in many diseases. There have, for example, been reports for many years that membrane organization and order might be altered in several cancers[88], but it remains unclear whether such changes play a part in disease progression or are merely byproducts of other metabolic changes. Other reports suggest that statins, which are now among the most widely prescribed drugs, may have uses in cancer chemotherapy[89], and they may alter endothelial cell function and suppress some inflammatory responses. It is likely that many of these effects are not directly related to their effects on cholesterol but are related to changes in other molecules, such as isoprenoids, that share the same initial biochemical synthetic steps as cholesterol[90]. Isoprenyl groups are important for anchoring several regulatory GTPases such as Ras and Rho in the membrane, and many of these pleiotropic effects of statins may be a consequence of changes in signalling pathways that use these GTPases.

The role of cholesterol and lipids in Alzheimer's disease has been actively studied for over a decade, on the basis of the observation that there is a genetic linkage between age of onset of Alzheimer's disease and the presence of the ε4 allele of apolipoprotein E (ApoE). Polymorphisms in other proteins involved in cholesterol metabolism may also have a genetic linkage with this disease[7]. ApoE is one of the main carriers of cholesterol in the brain, and it seems possible that alterations in cholesterol distribution or levels might have a role in formation of amyloid deposits. The amyloid in Alzheimer's disease is formed by aggregation of a 39–42-residue peptide, the Aβ peptide, which is formed by two proteolytic cleavages of a transmembrane protein, the amyloid precursor protein (APP). These cleavages take place in intracellular organelles. In tissue culture studies, severe lowering of cellular cholesterol (more than 35% reduction) partly inhibited the formation of the Aβ peptide, but moderate reduction in cellular cholesterol increased the formation of Aβ peptide[91]. Furthermore, rodents treated with statins can have increased amyloid production[91], and recent studies indicate that treatment with statins does not reduce the amyloid burden in humans[7]. Nevertheless, statins may be neuroprotective, perhaps because of their pleiotropic effects on endothelial cell function and as suppressors of inflammation[7,90]. There is still not a good mechanistic explanation for the association of the ε4 allele of ApoE with age of onset of Alzheimer's disease.

Future work

In the past several years there has been increased interest in the role of the lipids in biological membranes. The role of lipids and sterol derivatives as signalling molecules and second messengers is well established, but important new discoveries on the signalling roles of these molecules continue to be made. In this review, we have focused on the more subtle role of lipids and cholesterol in regulating the biophysical properties of membranes and how this affects cell physiology. Recent evidence points to the existence of coexisting microdomains within a single membrane, especially the plasma membrane, even though many important properties of these microdomains remain poorly characterized. These domains are important for regulating some signalling pathways, and we are beginning to understand how this may work in a few cases. Much work needs to be done to better characterize the biophysical properties of biological membranes and the effects that these properties have on membrane proteins.

Cells and organisms have developed extraordinarily sophisticated mechanisms for controlling the lipid composition, and hence the properties, of biological membranes. This control is based on regulating free cholesterol levels and also properties such as the degree of saturation of fatty acids. In atherosclerosis we have one clear example of what goes wrong when these homeostatic regulatory mechanisms are overwhelmed. In both the early and late stages of atherosclerosis there is evidence that changes in membrane bilayer properties influence disease progression. The roles of changes in bilayer properties in other diseases such as Alzheimer's or type II diabetes/metabolic syndrome is less clear, but this may be an area for significant new discoveries of disease mechanisms and treatments. ■

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