Membrane Cholesterol and the Formation of Cholesterol Domains in the Pathogenesis of Cardiovascular Disease

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Summary: At least three types of cholesterol-rich membrane domains have been described in biological membranes including cholesterol rafts, membrane caveolae and crystalline cholesterol domains,. While clear biological functions have been ascribed to both rafts and caveolae, little attention has been directed to the biological consequences of cholesterol enrichment of cell membranes and the formation of cholesterol domains. Elevated blood cholesterol levels have been shown to result in the enrichment of the cell plasma membrane with cholesterol in arterial smooth muscle cells (SMC), endothelial cells (EC) and cardiac myocytes. In the early period of cholesterol feeding (within days), the cell membrane enriches with cholesterol and membrane viscosity and membrane bilayer width increase. This latter effect severely alters membrane protein function, and recent data indicates that this induces the modulation of vascular cells (SMC and EC) to the atherosclerotic phenotype. In cardiac myocytes these membrane modifications appear to induce alterations in gene expression patterns that lead to the development of a heart failure phenotype. In addition, as the cholesterol content increases, phase separation of cholesterol occurs resulting in the formation of immiscible cholesterol domains within the membrane. These domains likely initiate nucleation of cholesterol crystals which would explain the origin of "cholesterol clefts" in atherosclerotic lesions. Taken together, these membrane alterations secondary to cholesterol enrichment constitute a "membrane lesion" which contribute to the very early pathogenic events underlying major human diseases including coronary artery disease, stroke and heart failure.

Keywords: atherosclerosis; cardiac myocytes; endothelium; heart failure; smooth muscle

Introduction

At its most basic level, the cell's plasma membrane serves the essential task of keeping the inside of the cell in and the outside of the cell out. To accomplish this nature has evolved a lipid bilayer envelope to surround the cell, into which a variety of proteins are inserted. With the lipid phase

 DOI: 10.1002/masy.200550104

impermeant to aqueous media, the protein components primarily confer selective and activatable translocation features to the membrane, giving the cells a dynamic capacity to respond appropriately to the environment and signals in it. The majority of membrane research activity has been directed to understanding the operation of the proteins in the membrane, with a significantly smaller effort directed toward understanding the lipid envelop. More recently, however, there has been a shift in interest toward the lipid envelop, driven largely by the recent discoveries of membrane lipid domains, especially rafts and caveolae. Singer and Nicholson (29) first proposed the modern view of the membrane as a phospholipid bilayer as a uniform two dimensional solvent system in which membrane proteins freely floated in a fluid sea of phospholipids. In addition to phospholipids, the membrane also contains substantial amounts of unesterified (free) cholesterol, up to nearly half the total lipid. Rather than being a homogenous mixture of lipids, however, we now know the membrane contains a variety of lipid domains with asymmetric distribution of phospholipids and cholesterol among and between the two membrane leaflets. Currently, its most intriguing features are the various lipid domains which include rafts and caveolae, and several excellent reviews have been published on these structures (20, 21, 28, 30). Less well known are the recently discovered crystalline cholesterol domains which exist in the plane of the membrane and the events leading to their appearance (35). Accordingly, this review focuses on the development of these domains, beginning with phenomenon of biological increases in membrane cholesterol, how this effects membrane structure and cell function, and the obligate phase separation of cholesterol from the phospholipids that leads to the formation of cholesterol domains within the membrane.

The Role of Cholesterol in the Cell Membrane

A. Permeability. In eukaryotes, cholesterol is contained in all cell membranes, but it is present in the highest concentration in the cell's plasma membrane (14). To the average scientist, cholesterol is generally thought to be a benign and inert structural lipid that "sets" membrane fluidity at some level appropriate for the specific membrane and cell type. Indeed there is some truth to this, but setting the fluidity may not be a primary function. Instead, studies have shown that when membranes lack cholesterol they tend to leak considerably, i.e., both water and small molecules (i.e., sizes up to sugars) freely pass. This is thought to result from the transient formation of fractional free volumes across the bilayer leaflets that result from the rotation of the fatty acyl

chains of neighboring phospholipids. At various points in time during their rotation, transient gaps or openings appear across the leaflet when the free ends of neighboring acyl chains are at their greatest distance from each other. When this happens simultaneously on both sides of the bilayer, a "fractional free space" forms that briefly allows water and small molecules to penetrate the bilayer (32, 33). The formation of these transient spaces is random and occurs all over the cell membrane. Adding cholesterol to the membrane restricts motion of the fatty acyl chains, condenses the fatty acyl chain region (hydrocarbon core) and greatly decreases membrane permeability. Thus, an essential role for cholesterol in the plasma membrane is to make this membrane particularly impermeant to water and charged particles. This explains why the plasma membrane contains most of the cell's cholesterol load (14). One of the byproducts of this effect of cholesterol is an obligate decrease in membrane fluidity since fluidity is driven by the ability of phospholipid fatty acyl chains to rotate freely.

B. Membrane width. Another byproduct of the presence of cholesterol in the membrane was discovered accidentally in our laboratory in collaborative studies using small angle x-ray scattering (SAXS) analysis (5). The original intent was to determine where in the membrane excess cholesterol resides. In this study a microsomal membrane pellet enriched with plasma membranes was freshly prepared from aortic smooth muscle cells (SMC) isolated from rabbits fed a high cholesterol diet for varying durations. Subjecting these membrane pellets to SAXS analysis revealed an increase in the cholesterol content in the bilayers spanning a dimension extending approximately 9 to 15 Å out from the center of the bilayer (Fig. 1), essentially similar to data obtained from NMR studies (16). Surprisingly, we also found that the phosphorous head-groups spread apart as the cholesterol content increased (Fig. 1 and Fig. 2). This suggested that increasing the cholesterol content of the membrane increased bilayer width. The absolute amount of increase in width is difficult to ascertain since the *d*-space increased approximately 4Å and 5.6 Å while the phosphorus-head-group separation increased 2.5Å, and 4.0Å at 8 and 10 weeks respectively on the cholesterol diet and (Fig. 2). The larger expansion in *d*-space measurement is likely due to hydration differences rather than absolute differences in membrane thickness (22, 34). In our studies, all data were obtained from samples studied at 97% RH and 37° C in order to minimize variations in *d*-space. Phosphorus head-group separation, on the other hand, is less affected by hydration and likely gives a better estimate of the relative differences in membrane thickness observed in membranes isolated at different times on the cholesterol diet. Clearly, the

data clearly support the concept that increases in membrane cholesterol content, either in vivo or in vitro, have a membrane "swelling" effect. Note also the tight correlation between membrane cholesterol content and phosphorus head-group spacing as well as *d*-space. Taken together, these data are consistent with a cholesterol-induced increase in bilayer width, as has been empirically demonstrated (18) and implied (23) previously in model systems. This increase in membrane cholesterol content correlated with time on diet, and membrane width correlated with membrane cholesterol content. It is important to note that these data were obtained in membranes that were enriched with cholesterol in vivo, i.e., in rabbits fed a cholesterol-rich (0.5%) diet. This effect of cholesterol on membrane width is therefore a natural biological phenomenon. Moreover, it appears to be a generic effect on phospholipid bilayers since increasing the cholesterol content increases bilayer width in a concentration-dependent fashion in synthetic phosphatidylcholine (PC) bilayers with either a uniform (DMPC) or a biological mixed (BCPC) fatty acyl chain population (Fig. 3).

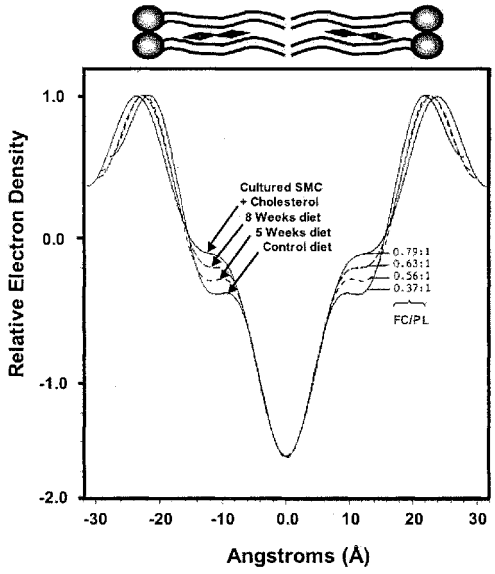

Fig. 1. Electron density profiles of a series of membranes (97% RH, 37°C) isolated from aortic SMC freshly obtained from rabbits maintained on a diet enriched with 0.5% cholesterol for 0, 5 and 8 weeks compared to cultured aortic SMC enriched with exogenous cholesterol. Reproduced from ref.(5).

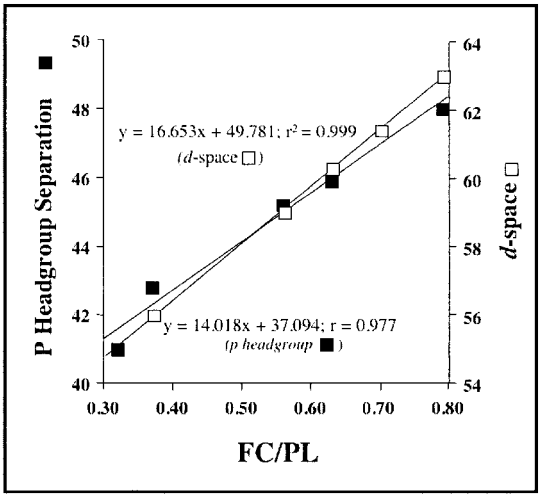

Fig. 2. Membrane bilayer width assessed by *d*-space and phosphorus head-group separation (Å) correlates ($p<.001$) with increasing membrane cholesterol content, and time on the high cholesterol diet. Cells were enriched with cholesterol in vivo or in cell culture (97% RH, 37°C). Redrawn from ref. [Chen, 1995 #13].

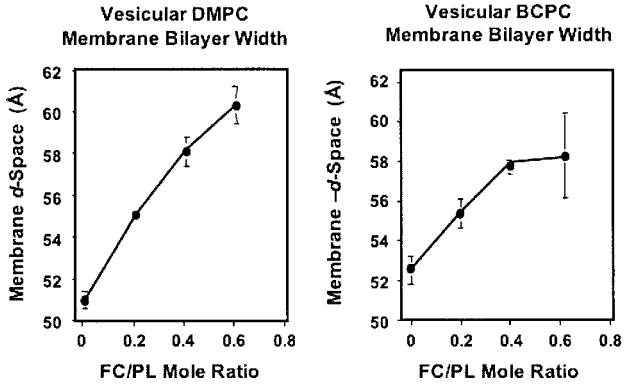

Fig. 3. A. Membrane width increases in a concentration-dependent fashion with increasing cholesterol content in model membranes (35). The left panel illustrates this effect in membranes made with dimyristoylPC (DMPC). The right panel illustrates this effect in membranes made with bovine cardiac PC (BCPC) which contains a biologic mixture of hydrocarbon chains. All samples studied at 97% RH, 37°C. Reproduced from ref. (35) with permission.

Table 1. Phenotypic changes in SMC after cholesterol enrichment *in-vitro* using liposomes (48 hours), *in-vivo* by cholesterol feeding (10 weeks), and the reversal of *in-vivo* effects using human HDL_3.

Phenotypic Alterations in SMC	Induced *in-vitro* by cholesterol	Induced *in-vivo* by cholesterol feeding	Reversal by HDL
1. ⇑ Membrane cholesterol content	√	√	√
2. ⇑ Membrane bilayer width	√	√	√
3. ⇑ Calcium permeability	√	√	√
4. ⇓ ATP-dependent K^+ efflux	√	√	--
5. ⇓ Na^+/K^+ ATPase activity	√	√	√
6. ⇑ Cytosolic calcium levels	√	√	√
7. ⇑ Vasoconstriction	√	√	--
8. ⇑ SMC proliferation	√	√	√
9. ⇑ Mitogen secretion	√	--	√
10. ⇑ Collagen synthesis	√	√	√

Interestingly, the increase in membrane cholesterol content and subsequently membrane width preceded the appearance of atherosclerotic lesions in cholesterol-fed animals by several weeks, suggesting a causal effect of altered membrane structure on the genesis of atherosclerotic vascular disease. We also assessed functions perturbed by cholesterol enrichment (Table 1), focusing on the activity of a variety of membrane proteins including Na^+/K^+ATPase, L-type calcium channels, ATP-dependent K^+ channels. All were disturbed by cholesterol enrichment. Supporting the idea that these alterations were indeed secondary to cholesterol enrichment, all were reversible by reducing membrane cholesterol back to normal using human HDL (HDL_3) as an acceptor (5) The implication that cholesterol directly affects bilayer width is profound. It is well established that the cell's plasma membrane has the greatest cholesterol content (14), as well as the greatest width (2). The width of various cell membranes is estimated accurately from protein hyrophobicity maps, and since the membrane-spanning domains of the various membrane proteins is quite precise in dimensions, it is reasonable to conclude that a particular membrane requires a prespecified width in order to accommodate the specific proteins targeted to that membrane. Accordingly, alterations in membrane cholesterol content, and thus membrane width, would be expected to have major effects on the function of important membrane proteins as we, and others have shown for the membrane-bound Na^+/K^+ATPase (3, 5, 38), the L-type calcium channel (1, 7), the ATP-dependent K^+ efflux channel (24), and the chloride channel (17). In fact, every membrane protein we have examined so far is altered by cholesterol enrichment. Thus,

cholesterol appears to have a dual role in the plasma membrane, one of maintaining the bilayer impermeant while simultaneously setting a specific, precise and unique bilayer width.

The Role of Membrane Cholesterol in Cardiovascular Disease

A. Vascular pathology. The above studies clearly demonstrate that excess membrane cholesterol content has major effects on membrane structure and function. Considering that membrane cholesterol content in SMC increases in vivo with dietary hypercholesterolemia (5), as do endothelial cells (15) and cardiac myocytes (9), the question of a pathogenic effect of excess membrane cholesterol becomes compelling. For example, in SMC cholesterol enrichment induces the modulation of these cells from the healthy quiescent phenotype to the atherosclerotic (fibroproliferative) phenotype as reflected by the cholesterol-induced increase in SMC proliferation, collagen synthesis and lesion formation (11). Notably, all these alterations can be reversed by amlodipine, a drug which restores membrane bilayer structure to back to normal and inhibits the development of atherosclerotic lesions (12). In these studies, while amlodipine restored the membrane, it did so without affecting membrane cholesterol content or fluidity, suggesting that the primary alteration in the membrane was structure, not fluidity. Cholesterol enrichment of EC also induces their modulation from the healthy quiescent phenotype to the atherosclerotic (inflammatory and adhesive) phenotype as reflected by the cholesterol-induced activation of NF-kB (15) and/or AP-1 (39) plus monocyte adhesion secondary to ICAM and VCAM expression. To be sure, this view of excess membrane cholesterol as atherogenic fits nicely into the reverse cholesterol transport role for HDL in reducing risk for atherosclerotic syndromes by reducing tissue and cell levels of cholesterol.

Another interesting and novel effect of excess membrane cholesterol potentially contributing to atherogenesis occurs when the cholesterol content of membranes exceeds saturation levels. This occurs in vivo when cholesterol-fed rabbits are maintained on diet beyond 8 weeks. Notably, SAXS analysis reveals phase separation of cholesterol in the membrane as suggested by the appearance of unexpected, highly coherent Bragg's peaks (1' and 2'; Fig 4 top right) (35). Unlike the expected Bragg's peaks with a calculated *d*-space of approximately 56Å (Fig 4 top left), the new peaks had a calculated *d*-space of 34Å. Assuming a cholesterol long axis of 17Å, we concluded that the new Bragg's peaks likely represent diffraction from immiscible tail-to-tail

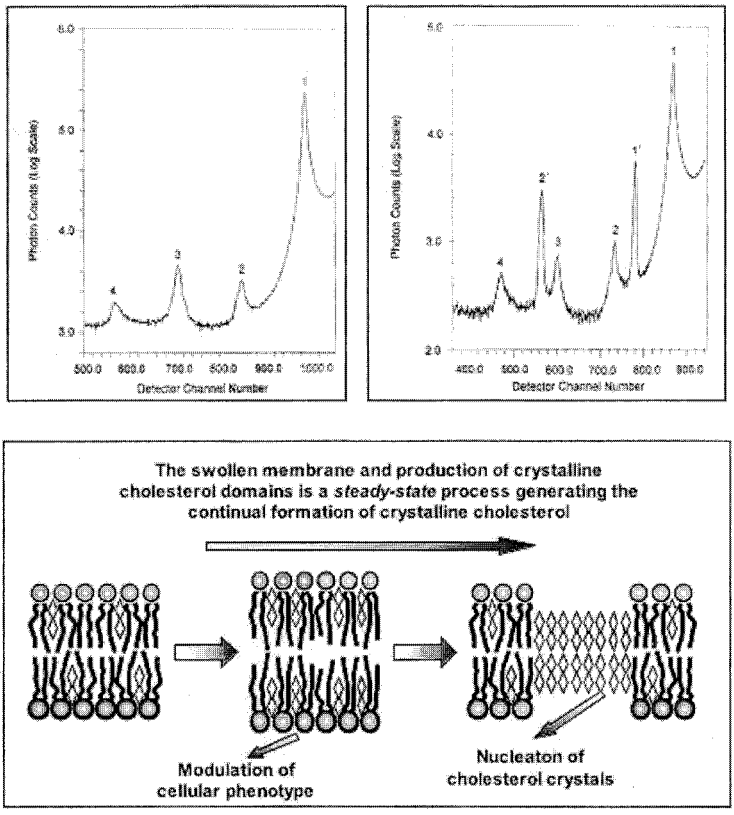

Fig. 4. Top panel shows typical Bragg SAXS peaks obtained from aortic SMC membranes isolated from control animals (left) and animals fed cholesterol for 10 weeks (right) (RH 97%; 37°C) (35). The bottom panel depicts our model of the structural changes in the membrane bilayer with increasing cholesterol concentrations and its consequences. Reproduced from ref. (35) with permission from the publisher.

oriented crystalline cholesterol domains. Support for the presence of these structures within the plane of the membrane (Fig. 4 bottom) comes from studies in which heating from 37° C to 45° C melted these structures with a concomitant increase in bilayer width (35). Likewise, cooling the bilayers back to 37° C resulted in the re-formation of the cholesterol domains as evidence by the reappearance of the 1' and 2' Bragg peaks. Ruocco and Shipely (25) reported similar tail-to-tail oriented crystalline cholesterol domains previously in synthetic membrane bilayers. Our

discovery of these structures was the first to document their formation and existence in vivo. Interestingly, Jacobs, et al., (10) later reported similar immiscible cholesterol domains in the lens of the human eye, a tissue whose membranes are highly enriched with cholesterol, further supporting their presence in nature. The finding of immiscible cholesterol domains in membranes of vascular cells was not expected. Their existence, however, may explain a previously unanswered question regarding the origin of crystalline cholesterol "clefts" in atherosclerotic lesions (Fig 5 top) that were described in the medical literature over 70 years ago. Light microscopy typically reveals needles of cholesterol deep inside atherosclerotic plaques in the coronary and peripheral arteries. Since enrichment of the cell membrane with cholesterol is likely a continuous, steady-state condition in hypercholesterolemic subjects, the immiscible cholesterol domains could serve as a site for the continuous nucleation of crystalline cholesterol in atherosclerotic vessels. These crystals have been observed forming inside as well as outside of cells. A similar mechanism has been suggested to be the origin of gall stones (36), structures well known to be rich in unesterified cholesterol. The association of numerous macrophages with the microscopic crystals (Fig. 5B left) suggests that they aggravate lesion formation in affected vessels by attracting inflammatory cells. While the evidence presented above suggests that excess membrane cholesterol may be an important contributor to atherogenesis, is important to point out that this view is clearly forged well "outside the box" of contemporary thinking. While hypercholesterolemia has been long known to induce the development of atherosclerotic lesions in humans and a variety of animal models, the cellular basis for this action virtually ignores the molecule cholesterol. Instead, it attributes atherogenesis to the formation of oxidized LDL (oxLDL) and oxidative injury to the endothelium (31) and SMC. In this context, blood cholesterol therefore is generally viewed as a "marker" for LDL levels and thus oxLDL-mediated oxidative stress. While there appears to be little doubt that oxidative stress plays an important role in atherogenesis, we provide data in this paper consistent with the suggestion that cholesterol enrichment of SMC may contribute importantly to the cellular events that initiate early cellular derangements leading to the development of atherosclerotic lesions. The concept of an atherogenic stimulus secondary to excess membrane cholesterol may identify a redundant pathway to atherogenesis independent of oxidative stress. Accordingly, this may explain the relative ineffectiveness of supplemental dietary antioxidants to provide atheroprotection in human studies (8, 19, 40). On the other hand, the ineffectiveness of anti-oxidant therapy to

prevent the development of atherosclerotic lesions in humans may indicate that oxidative stress does not play a central or pivotal role in the pathogenesis of this disease. In this case, a role for excess membrane cholesterol in atherogenesis may be a central issue.

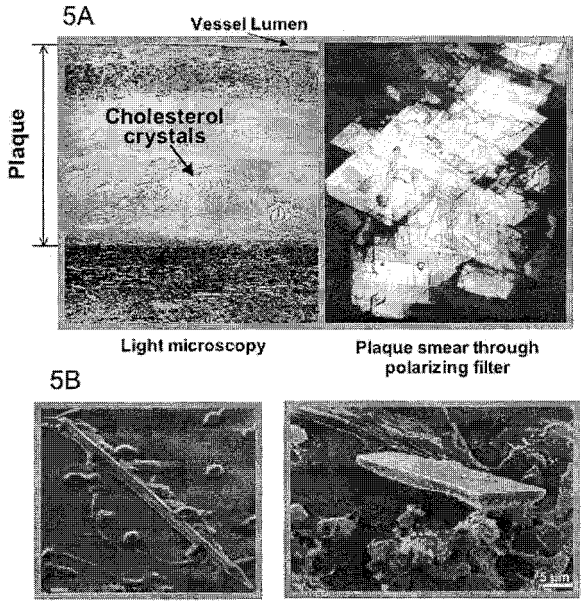

Fig. 5. A. Left panel shows the presence of crystals of cholesterol ("cholesterol clefts") that typically appear in mature atherosclerotic lesions shown under light microscopy of a section of human coronary artery obtained at post-mortem. Right panel shows the crystalline nature of this material when it is spread on a cover slip and viewed under polarizing microscopy. Reproduced from ref. (26) with permission from the publisher. B. Formation of cholesterol crystals in a cultured macrophage cell line enriched with cholesterol. Note the attraction of monocytes to the crystal in the left panel. Reproduced from ref. (12) with permission from the publisher.

B. Cardiac pathology. The heart disease (cardiomyopathy) typically associated with elevated blood cholesterol levels has always been assumed to be strictly limited to coronary artery disease, i.e., an ischemic condition (myocardial ischemia) of the heart muscle (myocardium) which occurs following a sudden occlusion of a coronary artery due rupture of an atherosclerotic plaque. There is no question that people with elevated cholesterol levels are at greatest risk for acute myocardial infarction. We have recently shown in rabbits however, that as blood cholesterol levels rise, membrane cholesterol content in the cardiac myocyte rises simultaneously (Fig. 6A), an effect

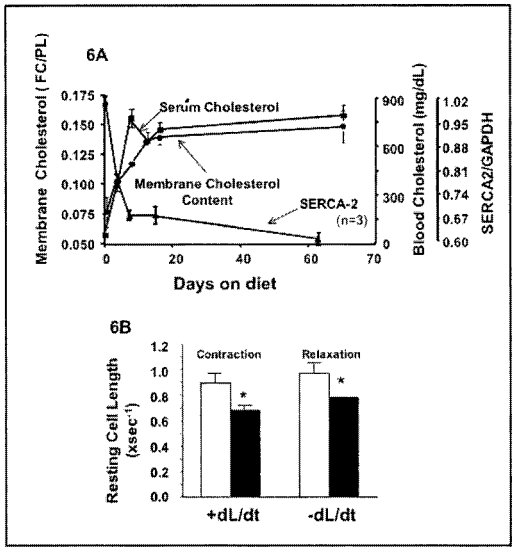

Fig. 6. A. Time course of increase in blood cholesterol in rabbits following the initiation of cholesterol feeding (0.5%). Note the parallel increase in blood and membrane cholesterol content of the cardiac myocyte, and the reciprocal fall in SERCA 2 mRNA gene expression levels.
B. Impaired systolic (contraction) and diastolic (relaxation) function in single left ventricular myocytes freshly isolated from rabbits fed a cholesterol-rich diet (solid bars) compared to control diet (solid bars). Reproduced from ref (9) with permission from the publisher.

that is accompanied by contractile dysfunction (Fig. 6B), i.e., heart failure (9). The contractile impairment is an intrinsic cellular defect which we termed the "cholesterol cardiomyopathy," and is secondary to cholesterol enrichment of the myocyte membrane. This cardiomyopathy is virtually unrelated to any atherosclerotic processes. This was apparent since mRNA expression levels for SERCA 2, a major intracellular calcium regulatory enzyme fell in parallel with the increase in blood and myocyte membrane cholesterol levels, i.e., within only 4 days after the initiation of feeding the cholesterol diet and weeks before arterial disease develops (Fig. 6A). SERCA 2 is a calcium transport enzyme that pumps calcium from the cytosol into the sarcoplasmic reticulum (SR) and thus participates in regulating cardiac contraction and relaxation, both of which were impaired in rabbits fed the high cholesterol diet (Fig 6B). While the magnitude of cardiac dysfunction was mild, it has been shown that hypertension, the leading

cause of heart failure in humans, impacts the myocardium to a much greater degree in the presence of hypercholesterolemia than in its absence (37). Considering that over 37 million Americans have hypertension in combination with hypercholesterolemia, this may be an important concern. That the cholesterol cardiomyopathy may exist as a clinical entity in humans is supported by the recent observations that statins, drugs that lower blood cholesterol levels, improve cardiac function in patients with heart failure (6, 13, 27).

Conclusion

No molecule has been more decorated than cholesterol, with no less than 13 Nobel Prizes awarded for studies directed toward understanding its complex chemistry and biology (4). Among its least understood actions are in cell function where it is largely thought of as an inert molecule that provides structural integrity to cell membranes. Studies from our laboratories clearly show that the molecule cholesterol is anything but inert; in fact, it's quite the opposite. Too much cholesterol in the membrane gives rise to a sequence of events that leads to phenotypic modulation to a disease phenotype. In vascular cells, excess membrane cholesterol induces modulation of the cells to the atherosclerotic phenotype, whereas in cardiac myocytes, this leads to a heart failure phenotype. Considering the biological consequences of excess membrane cholesterol, one would anticipate that rigid cellular controls would be in place to firmly hold membrane cholesterol at levels appropriate to preserve the integrity of the plasma membrane. On the contrary, however, membrane cholesterol is rather easily disturbed, both experimentally at the lab bench as well as in vivo in experimental animals as we have repeatedly shown. Moreover, there is considerable evidence that control of membrane cholesterol is equally fragile in humans as well, given the extent of vascular disease in the human population. Figure 4 (bottom) summarizes the potential roles of excess membrane cholesterol to form a "membrane lesion" which initiates and maintains membrane swelling and its progression to the formation of immiscible cholesterol domains. A compelling question now is how excess membrane cholesterol and its effects on membrane structure mediate the rapid changes in gene expression patterns that we have observed. Upstream signaling is likely involved, but how this is accomplished is not clear and constitutes an important area for future studies.

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