

Cholesterol crystals piercing the arterial plaque and intima trigger local and systemic inflammation

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Abstract: The response to arterial wall injury is an inflammatory process, which over time becomes integral to the development of atherosclerosis and subsequent plaque instability. However, the underlying injurious agent, critical to this process, has not received much attention. In this review, a model of plaque rupture is hypothesized with two stages of inflammatory activity. In stage I (cholesterol crystal-induced cell injury and apoptosis), intracellular cholesterol crystals induce foam cell apoptosis, setting up a vicious cycle by signaling more macrophages, resulting in accumulation of extra cellular lipids. This local inflammation eventually leads to the formation of a semi-liquid, lipid-rich necrotic core of a vulnerable plaque. In stage II (cholesterol crystal-induced arterial wall injury), the saturated lipid core is now primed for crystallization, which can manifest as a clinical syndrome with a systemic inflammation response. Cholesterol crystallization is the trigger that causes core expansion, leading to intimal injury. We recently demonstrated that when cholesterol crystallizes from a liquid to a solid state, it undergoes volume expansion, which can tear the plaque cap. This observation of cholesterol crystals perforating the cap and intimal surface was made in the plaques of patients who died with acute coronary syndrome. We have also demonstrated that several agents (ie, statins, aspirin, and ethanol) can dissolve cholesterol crystals and may be exerting their immediate benefits by this direct mechanism. Also, because recent studies have demonstrated that high-sensitivity C-reactive protein may be a reliable marker in selecting patients for statin therapy, it could reflect the presence of intimal injury by cholesterol crystals. This was demonstrated in an atherosclerotic rabbit model. Therefore, we propose that cholesterol crystallization could help explain in part both local and systemic inflammation associated with atherosclerosis.

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Inflammation is defined as a physiologic response to injury that attempts to heal the incurred damage.¹ However, this response may be exaggerated and/or protracted, then itself becoming the source of aggravated injury. This paradox has been recognized in a variety of medical conditions. Perhaps the most challenging aspect of this process is

determining whether the inflammation is the cause or result of the injury and if treating the inflammation per se would be an effective approach in controlling the disease. This review will attempt to define the process leading to atherosclerotic cardiovascular disease and its clinical manifestations. Future investigations that may help elucidate its dynamics are proposed.

The role of inflammation in atherosclerosis had been implicated as far back as two centuries ago, when Rudolph Virchow (1821–1902) coined the term *endarteritis*

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deformans in describing atherosclerosis and proposed that inflammation was the underlying cause.² More recently, Russell Ross (1929–1999) proposed the concept of inflammation in response to vascular injury. This concept has attracted more attention because various inflammatory markers were found to be involved in atherosclerosis.^{3,4} Thus, the role of inflammation is now considered to be integral in atherogenesis. However, an equally important aspect of this entire process is identifying the factors and mechanisms that cause the vascular injury. In the 1970s, Donald Small⁵ evaluated cholesterol crystals and their potential role in atherosclerosis. However, the concept of plaque rupture as the underlying cause of acute cardiovascular events had not been elucidated at that time, and hence the connection between cholesterol crystals and plaque rupture was unrecognized.

Recently my group^{6,7} demonstrated that when cholesterol crystallizes from a liquid to a solid state, it expands in volume (Fig. 1). Moreover, it has already been shown that cholesterol is present in a liquid state in the arterial wall.⁸ Therefore, expansion within the confined space of the necrotic core of atheromatous plaques can lead to disruption of the plaque cap and overlying intima, leading to arterial thrombosis. In *ex vivo* studies,⁹ we demonstrated that cholesterol crystals were densely clustered at sites of plaque rupture in coronary and carotid arteries from

patients with acute cardiovascular or neurological events, respectively (Fig. 2). This observation was made possible by avoiding the use of solvents in the tissue preparation process for scanning electron microscopy. Our finding of cholesterol crystals perforating the intimal surface raises the question as to whether there is a link between this injurious process and inflammation.

Evidence from several clinical trials has demonstrated that inflammatory markers are predictive of future acute cardiovascular events.^{10–16} High-sensitivity C-reactive protein (hs-CRP) has been the most studied marker, and it was recently used in the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study to select patients for statin treatment.¹⁷ The results demonstrated a dramatic reduction in cardiovascular events favoring the statin treated cohort based on elevated hs-CRP levels. Thus, hs-CRP may be a marker for underlying vascular injury.^{18,19}

Considering a cholesterol crystal-mediated intimal injury the potential for an inflammatory response would be expected. Therefore, such a scenario of injury and inflammation would fit into the clinical paradigm of inflammatory markers and their signaling of vascular injury by cholesterol crystals. We have demonstrated that statins, alcohol, and aspirin all have an ameliorative effect on cholesterol crystal formation while reducing inflammation and

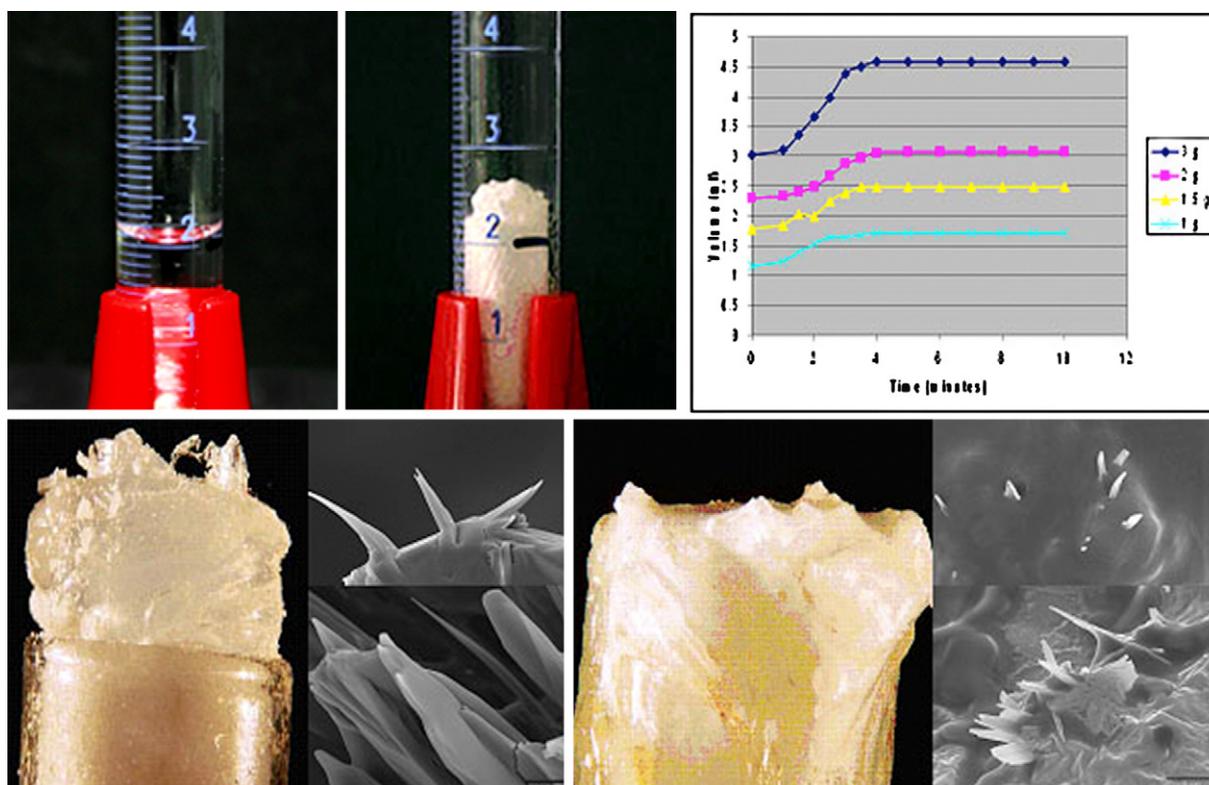


Figure 1 Demonstration of volume expansion of cholesterol during crystallization. (Upper panel) Dissolved cholesterol in graduated cylinder expands in volume above the meniscus line. Graph of volume expansion is greater with increasing amounts of cholesterol (1–3 g). (Lower panel) Cholesterol crystals are seen growing above the edge of the test tube after crystallization. Scanning electron micrographs demonstrate sharp-tipped crystal geometries. When a fibrous membrane is placed over the mouth of the test tube, crystals perforate the membrane as noted by scanning electron microscopy (bar = 5 µm). Reproduced with permission.^{6,7}

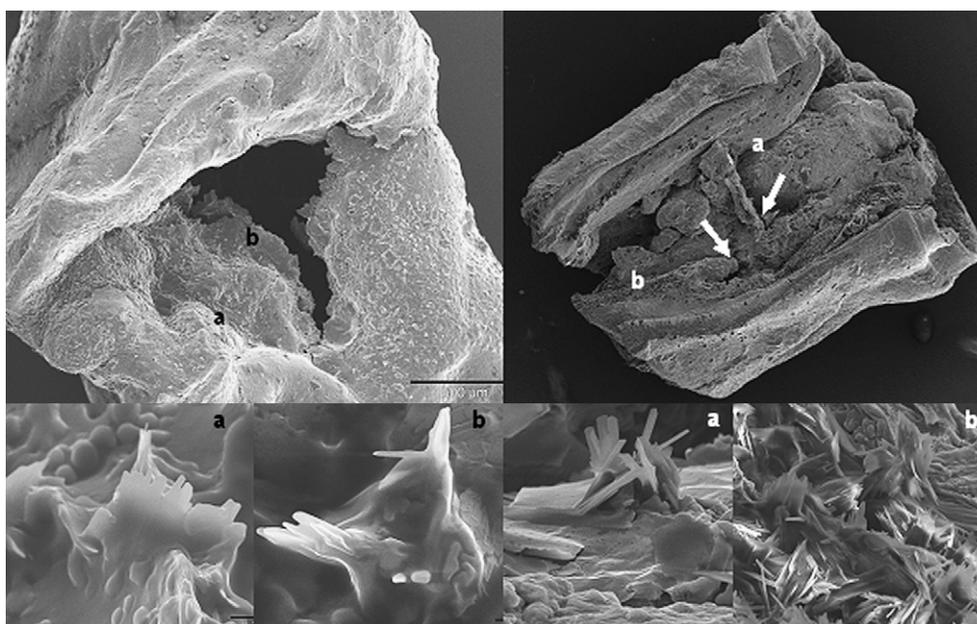


Figure 2 Cholesterol crystals perforating the intima in a patient who died of acute coronary syndrome. (Left column) left anterior descending artery just below the site of plaque rupture demonstrating cholesterol crystals perforating the intima (bar = 500 μ m). (Right column) Cholesterol crystals perforating the plaque cap and intima (bars = 2, 5 μ m) at site of plaque rupture (arrows: torn cap edges; Bar = 1 mm). Reproduced with permission.^{7,9}

providing protective effects from acute events.^{20–34} This finding lends additional support to the concept that cholesterol crystals could be triggering a systemic inflammation response by vascular injury.

Triggers of vascular inflammation

Several agents and physiological conditions have been proposed as risk factors for vascular injury, including diabetes, hypertension, smoking, and elevated serum cholesterol.³⁵ All these risk factors seem to induce endothelial dysfunction and accelerate atherogenesis. Moreover, it has been well documented that cholesterol is an important player in this process and that its reduction is highly protective from cardiovascular events.^{36–41} The primary pathological lesion underlying risk for cardiovascular events is the vulnerable atherosclerotic plaque. This plaque has been defined by histology to have a lipid-rich necrotic core and a thin fibrous cap with weakened structural support and the presence of cellular inflammation.^{42–44} Recently, the presence of vulnerable plaques has been confirmed in the Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT) trial.⁴⁵ The relationship between elevated serum cholesterol and the underlying pathology has been widely investigated, but the exact mechanism of plaque rupture has been elusive. Our observation of cholesterol crystals expanding may help elucidate the role of inflammation in this pathology. On the basis of the process of cholesterol crystallization, the role of inflammation in plaque rupture appears to occur in two stages—the first is in the formation of the vulnerable

plaque, and the second is in the systemic response to plaque disruption.

Stage I (cholesterol crystal-induced cellular injury and apoptosis)

The localized cellular inflammation results in the formation of the lipid-rich necrotic core, the major hallmark of a vulnerable plaque (Fig. 3). The process is initiated by entry of low-density lipoprotein (LDL) into the arterial wall, resulting in entrapment caused by interaction with cellular matrix components, mainly glycosaminoglycans, which have high affinity to apolipoprotein B. Also, small dense LDL particles and genetically determined vascular affinity may be critical factors as well.^{46,47} This step is followed by entry of monocytes from the circulation transforming into macrophages that take up the LDL. As cholesterol builds up in the macrophages, they become foam cells that eventually form fatty streaks, leading to the development of the necrotic core.^{5,48–51} In the foam cells cholesterol crystallizes, triggering a danger signal that initiates a local inflammatory response via a Nod-like receptor, NLRP3 inflammasome protein.⁵² This process occurs very early in the development phase of atherosclerotic plaques.

The intracellular crystals can lead to death of the foam cells, contributing to a local extra cellular build up of cholesterol derived from both the cell membranes as well as their earlier imbibed cholesterol load.^{53,54} This material then forms the lipid-rich necrotic core, which once formed, becomes an accumulation of localized lipid intermixed with cellular debris often referred to as “the gruel.” As more

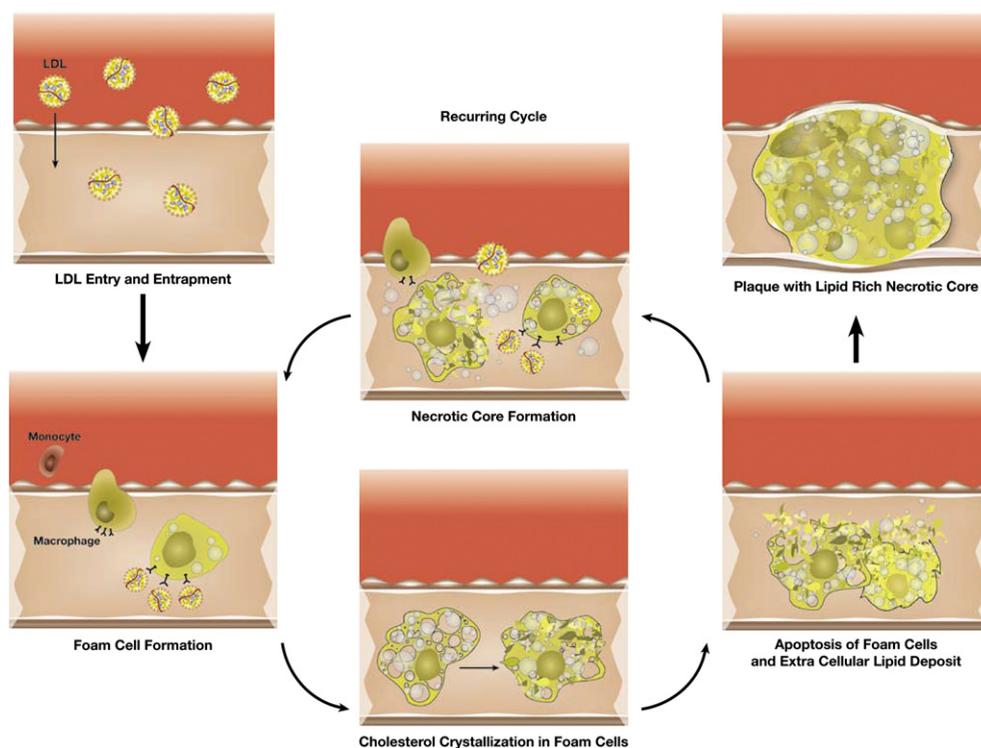


Figure 3 A vicious cycle of vascular inflammation with recurrent injury results in the formation of a necrotic core and eventually vulnerable plaque. The initial step is entry and entrapment of LDL within the arterial wall, followed by entry of monocytes that take up the LDL and become foam cells. Crystals in the foam cells induce apoptosis, leading to further attraction of macrophages that forms an extracellular lipid pool within the arterial wall. This then constitutes the basic elements of a vulnerable plaque.

macrophages are attracted to the site of inflammation by chemotactic signals, more lipid is taken up by those macrophages to form additional foam cells. Many of those macrophages then die and release their content, signaling more macrophages to the site thus setting up a vicious cycle that ultimately forms a saturated lipid pool. Also, red blood cells (RBCs) released from injured vasa vasorum feeding the plaque provide additional cholesterol from their cell membranes, leading to a greater cholesterol concentration and saturation.^{55–58} The vasa vasorum can also be injured by the expanding sharply tipped cholesterol crystals, releasing RBCs into the necrotic core. Moreover, the presence of free extracellular cholesterol in soft tissues has been identified as a cause of chronic inflammation.⁵⁹

Once the necrotic core is formed within the confined space of the arterial wall between the internal and external elastic lamina, it then becomes subject to the local physical and chemical forces. Consequently, saturation, temperature, pressure, and pH could individually or in combination trigger the crystallization of the cholesterol within the necrotic core. Several of these physical factors have already been shown *in vitro* to trigger cholesterol crystallization.⁶⁰ More specifically, hydration of the cholesterol molecule is one of the local factors that contributes to greater volume expansion. It is the cholesterol monohydrate species that has been identified by crystallography studies as the predominant cholesterol molecule present in human atherosclerotic plaques.⁶¹ Thus, any of these factors

individually or in combination could result in cholesterol crystallization, leading to plaque disruption. These observations also fit the unpredictable clinical presentations of acute cardiovascular syndromes that seem to increase with environmental stressors such as cold weather or physical exertion.^{62,63}

Stage II (cholesterol crystal-induced arterial wall injury)

This stage is a systemic inflammation generated by the compromise of the fibrous plaque cap. As demonstrated, crystallization of cholesterol leads to volume expansion of the necrotic core that can cause plaque rupture and/or erosion.^{9,64} This event then leads to the disruption of the plaque cap and overlying endothelium, triggering a systemic inflammatory response (Fig. 4). The local production of interleukin (IL)-6 molecule by lymphocytes occurs in response to intimal injury that then circulates to the liver and signals the production of hs-CRP, which is an acute-phase reactant.^{3,4,18,19}

Plaque rupture may occur suddenly or slowly on the basis of the size of the lipid pool. Large pools would tend to produce sudden rupture by rapid expansion, which is often seen in men, whereas smaller pools would tend to produce a slower, more protracted event, causing erosion as seen more often in women.^{64,65} We have demonstrated in an

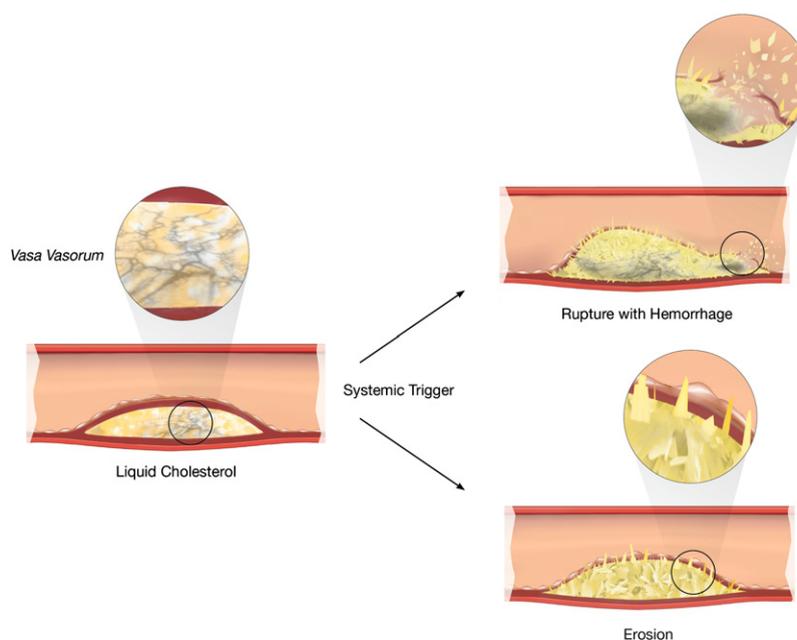


Figure 4 Mechanism of plaque rupture and/or erosion induced by cholesterol crystallization. In the case of a large necrotic core, the plaque cap is torn, leading to rupture, whereas in the case of a small necrotic core, it leads to erosion. Furthermore, trauma to the vasa vasorum by cholesterol crystals within the plaque causes intra plaque hemorrhage. Modified and reproduced with permission.⁶⁴

atherosclerotic rabbit model that continuous feeding of cholesterol-enriched diet leads to a progressive increase in hs-CRP, IL-6, and plasminogen activator inhibitor-1. However, once plaque rupture occurs, the biomarkers rise considerably higher.^{66,67}

The question then arises whether cholesterol crystals released from plaques was caused by a passive process associated with plaque disruption or the actual cause of the event. Several lines of evidence suggest that this was causative. First, cholesterol crystals were noted perforating the intima not only at the site of plaque rupture but in the adjacent arterial wall, indicating an active process involving locations beyond the rupture site (Fig. 2). Furthermore, there were multiple sites of plaque disruption in other coronary arteries.⁹ Second, in vitro studies have demonstrated that when a fibrous membrane is placed in the path of growing cholesterol crystals, the membrane is perforated and torn by sharp tipped crystals.^{6,7} Moreover, histology demonstrates the edges of ruptured fibrous plaque caps to be frayed, suggesting a dynamic snapping like tear. Third, in an atherosclerotic rabbit model of plaque disruption and thrombosis, the presence of cholesterol crystals was associated with thrombosis while their absence was not.⁶⁷ Fourth, patients who had severe atherosclerosis and died of noncardiac conditions did not have cholesterol crystals perforating the intimal surface.⁹ Only those who died with acute coronary syndrome had evidence of cholesterol crystals perforating the intima. Therefore, we hypothesize that this intimal injury leads to a systemic inflammatory response. Furthermore, our model of cholesterol crystal expansion would also explain the sudden growth of atherosclerotic plaques often recognized to occur in patients. This is observed on repeat coronary angiography in the same

patient where plaques lay quiescent for years and then suddenly crop up as would be expected from our observations of cholesterol crystallization with expansion.

Inflammatory markers associated with atherosclerosis

Various types of serum inflammatory markers have been associated with atherosclerotic cardiovascular disease, and the list grows steadily. Some of these include hs-CRP, IL-6, serum amyloid A, monocyte chemoattractant protein-1, myeloperoxidase, and lipoprotein-associated phospholipase A₂. Several cellular adhesion molecules have also been identified, including intercellular adhesion molecules. However, thus far, hs-CRP is the primary contender as a predictor of future cardiovascular events as well as short- and long-term mortality during acute coronary syndromes.⁶⁸⁻⁷¹

The JUPITER study used hs-CRP of >2 mg/liter to select patients for treatment with 20 mg of rosuvastatin.¹⁷ This measure provided a major reduction in acute cardiovascular events (44%). In consideration of our recent findings regarding the mechanism of plaque disruption, we propose that hs-CRP elevation represents early intimal injury by cholesterol crystals perforating the intima, thus providing a greater predictive value for future events than LDL alone.¹⁵

Mechanisms of plaque disruption

The current thinking is that plaque rupture occurs by weakening of the plaque cap from the release of metalloproteinases and collagenases that digest the fibrous

cap.^{72,73} These originate from inflammatory cell infiltration, that is, macrophages and lymphocytes. However, a direct link between plaque rupture and this process has not been established. Given our recent findings regarding cholesterol crystallization and volume expansion, we suggest that this mechanism is responsible for the final stage of plaque rupture. Local inflammation (stage I) is critical in the formation of the necrotic core but compromising and rupture of the plaque cap by expanding sharp-tipped cholesterol crystals triggers primarily a systemic inflammation (stage II). Even the hemorrhage within the plaque could be caused by the same process of cholesterol crystal expansion that can pierce through the vasa vasorum as illustrated in Figure 4.

Multiple sites of rupture in the same and other vascular beds have been noted by angiography, angioscopy, and intravascular ultrasound, suggesting a systemic process.^{74–78} We have also observed cholesterol crystals perforating the intima in several arterial beds in the same patient.⁷⁹ This may be attributable to a systemic process that can lead to local environmental change such as pH, temperature, cholesterol saturation, and hydration of the cholesterol molecules. These have all been tested and found to enhance cholesterol crystallization.⁶⁰ A systemic process can also lead to enhanced local inflammatory activity. These can influence the local milieu by activating the macrophages to become phagocytotic and increase their lipid uptake to make foam cells. Various systemic processes have been incriminated, including various types of infections.⁸⁰ A similar activation has been demonstrated *in vitro* where macrophages need to be stimulated in order to start phagocytosis.⁸¹ Eventually, this process will lead to crystal formation within the macrophages/foam cells, causing apoptosis. This then feeds the cycle of extra cellular lipid accumulation to form the vulnerable plaque.

Role of statins, ethanol and aspirin in plaque disruption

Statins, ethanol, and aspirin (ASA) have all been found to be protective of acute cardiovascular events.^{19–27} In all cases, we have found statins, ethanol, and ASA to be effective solvents of cholesterol crystals. In the context of our model, dissolving the cholesterol crystals provides protection from volume expansion and intimal injury reducing acute clinical events. Thus, dissolving the cholesterol crystals could explain some of the pleiotropic effects of statins and the dose relationship to enhanced effects beyond the scope of LDL lowering.⁸² Similarly, moderate ethanol consumption has been found to be protective, and we have already demonstrated that ethanol dissolves cholesterol crystals.^{7,9} Moreover, ASA is a lipophilic compound that may dissolve cholesterol crystals by a “like-dissolves-like” mechanism. Alternatively, the cholesterol molecule may be altered chemically when combining with these compounds to change its crystallization characteristics.

These potential mechanisms could explain the pleiotropic benefits of these compounds as well as their early and quick action that has been described in acute cardiovascular syndrome.^{20,83} The use of both statins and ASA has been shown to improve the immediate outcomes after interventional procedures especially during acute cardiovascular events.^{19–22,26,27} Also, both agents and ethanol have been shown to have anti-inflammatory properties that may be explained by the same process.^{31–33} Furthermore, this may explain how high-dose statins has been associated with a small-but-significant increase in hemorrhagic stroke because if cholesterol crystals are imbedded in the arterial wall at the site of the stroke and then dissolved, it could cause blood to leak at that site from “unplugged” holes in the arterial walls.⁸⁴ Moreover, cerebral arteries have only one layer of elastic lamina, making them potentially more fragile.

Future investigations

Systemic effects that lead to local plaque instability would be a major direction for future investigations. We have already demonstrated that cholesterol crystallization *in vitro* is enhanced by pH shifts, temperature, and saturation changes.⁶⁰ However, other aspects of this process need to be defined, including emotional and physical stress, febrile illness, and a prothrombotic state, to list a few. All these may be contributors to the final systemic state required to induce cholesterol crystallization that leads to an acute event. Also, showering of cholesterol crystals released by plaque rupture, causing end organ damage, would also be important to explore. Finally, various agents that can prevent formation or dissolve cholesterol crystals would be an important topic for future investigation.

Treatment of the inflammatory response with various cell growth-inhibiting agents such as paclitaxel may provide an approach to prevent or regress atherosclerotic plaques similar to what is achieved by drug eluting stents in preventing restenosis. An example of this approach has already been demonstrated in the atherosclerotic rabbit model.⁸⁵

Discussion and summary

Crystal-induced diseases have been well recognized over the centuries as a cause of illness. The most common include gout, renal, and gall stones, but more rare forms of crystals have been described as the result of genetic errors, leading to accumulation of certain proteins and/or molecules and causing crystal deposits in the cornea of the eye or connective tissues (ie, ochronosis), triggering inflammation.^{86–90} In gout, monosodium urate crystals have been demonstrated to trigger the danger gene signal to initiate an inflammatory response.⁸⁸ The same has now been confirmed for cholesterol crystals.⁵² Crystal formation and deposition in the confined space of a joint will trigger a systemic inflammation. Similar environmental conditions

are known to facilitate this including local trauma, dehydration and saturation of proteins. A similar scenario can be hypothesized for cholesterol crystal formation within the arterial wall. Sudden expansion within this confined space will lead to an “explosive” event by cholesterol crystallization when the ideal physical conditions have been established (ie, saturation, temperature and pH). The similarities between crystalloid diseases are common and point to the need for exploring the local milieu of arteries and how that could influence atherosclerosis plaque progression and instability. This review leads to the conclusion that cholesterol crystals are the most likely culprit in triggering both local and systemic inflammation leading to plaque rupture and thrombosis.

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