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News and Commentary

Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol

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Macrophages are the most prominent cell type in atherosclerotic lesions, and a portion of these cells become apoptotic, particularly in advanced lesions. One inducer of apoptosis in atherosclerosis is the accumulation of large amounts of intracellular unesterified or 'free' cholesterol (FC). A portion of the FC accumulates in the endoplasmic reticulum (ER) membrane, which is normally cholesterol poor and highly fluid. This event, probably by altering the function of integral ER membrane proteins, induces the ER stress signal transduction pathway, known as the unfolded protein response (UPR). A branch of the UPR eventually leads to an apoptotic response in the macrophages, which is mediated by both Fas and mitochondrial pathways. *In vivo* evidence suggests that this chain of events may promote plaque destabilization in advanced atherosclerotic lesions, which is ultimately responsible for acute atherothrombotic vascular occlusion and tissue infarction.

Atherogenesis, Acute Obstructive Vascular Disease, and Culprit Lesions

To gain a full understanding of the potential importance of macrophage apoptosis in atherothrombotic vascular disease, it is necessary to outline our current understanding of how atherosclerotic lesions develop and progress. By the teenage years, most people in industrialized societies have numerous deposits of fat, cells, and extracellular material in the intima of focal areas of the arterial tree. Most of these deposits, known as atherosclerotic lesions, remain asymptomatic throughout a person's life. Even those lesions that progress usually encroach upon the arterial lumen so slowly that compensatory mechanisms in the arterial wall, such as vascular remodeling and collateral vessel formation, maintain adequate blood flow. Moreover, in the face of risk factor improvement, some lesions can remain stable in size or even regress. However, in the face of continuing risk factor exposure, a small percentage of lesions – often referred to as 'vulnerable plaques' – may undergo a transformation resulting in lesional necrosis and plaque erosion or rupture. At the site of plaque disruption, circulating platelets are exposed to thrombogenic material, which can lead to acute thrombosis, acute vascular obstruction, and tissue infarction.[1] This chain of events is the cause of most cases of sudden cardiac death, myocardial infarction, stroke, and other acute obstructive vascular diseases, which collectively represent the leading cause of death in the industrialized world.

Given the magnitude of atherosclerotic vascular disease, it is essential that we understand how atherosclerotic lesions develop and, most importantly, how benign lesions are converted into vulnerable plaques. While much remains to be learned, an overall scenario can be pieced together based on data from human pathological analysis, animal models, and mechanistic cell-culture and *in-vitro* studies. The earliest and presumably initiating event in atherogenesis is the focal retention of circulating atherogenic lipoproteins, such as cholesterol-rich LDL and remnant lipoproteins, in the subendothelial space.[2,3] These retained lipoproteins then induce a series of biological responses that induce atherosclerotic lesion formation. Some of these responses probably occur only after the retained lipoproteins have been chemically or enzymatically modified in the intima, for example, by oxidation and aggregation.[4,5] The most prominent responses to retained lipoproteins are: (a) endothelial alterations, including induction of adhesion molecules; (b) recruitment of macrophages and lymphocytes to the intima, which in turn induces an inflammatory state in the arterial wall; (c) accumulation of cholesteryl fatty acyl esters in macrophages and, to a lesser extent, smooth muscle cells (so-called 'foam cell' formation); and (d) medial-to-intimal migration of smooth muscle cells, which then become transformed into matrix-producing cells that produce a fibrous cap over the lesion. As a result, subendothelial lesions characterized by lipids, hypercellularity, inflammation, and extracellular matrix develop.

As mentioned previously, most of these lesions remain clinically silent, but the small percentage of lesions that become unstable can be deadly. Here again, we can only speculate on the chain of events leading to vulnerable plaque formation based on observational studies. The most prominent features of vulnerable plaques include: (a) thinning of the fibrous cap that, as alluded to above, characteristically covers intermediate-stage atherosclerotic lesions;[6] (b) a 'necrotic' or 'lipid' core, consisting of dead macrophages, macrophage debris, and extracellular lipid, including needle-shaped cholesterol crystals;[6–9] (c) a heightened stage of inflammation, consisting of activated leukocytes and inflammatory cytokines;[10] and (d) intracellular and extracellular accumulation of unesterified or 'free' cholesterol (FC), which is in distinct contrast to the prominence of cholesteryl esters in earlier, more benign lesions.[11] While a causative relationship between any of these features and plaque vulnerability has

not been definitively established, it is likely that thinning of the fibrous cap directly promotes plaque breakdown and that the other features of vulnerable plaques outlined above contribute, in turn, to cap thinning. For example, the inflammatory milieu of advanced lesions may induce the secretion of matrix proteases by macrophages and promote the death of matrix-producing smooth muscle cells, both of which would promote cap thinning.[10] In another scenario, proteases and inflammatory molecules released from dying macrophages in the necrotic core also contribute to plaque destabilization.

According to this scenario, macrophage death in advanced atherosclerotic lesions is a key event in the conversion of benign lesions to vulnerable plaques.[12] That dead macrophages give rise to the 'necrotic' core of vulnerable plaques is indicated by immunohistochemistry data showing that the core debris is of macrophage origin.[9]

In and surrounding the core are macrophages that have not yet disintegrated, and many of the cells shows signs of both apoptosis and necrosis. Apoptotic cells have been identified by typical morphologic features, such as nuclear condensation, and they stain positively for both DNA fragmentation and activated caspases.[7,8] Of note, DNA fragmentation has been detected by a stringent variant of the TUNEL assay that avoids common artifacts.[8] Other macrophages have the morphological features of necrosis, notably swollen organelles and disrupted cellular membranes. While it is possible that this latter subpopulation of macrophages die by a completely nonapoptotic mechanism, it is likely that at least some originate from apoptotic macrophages that secondarily became necrotic due to lack of phagocytic clearance (i.e., secondary, or post-apoptotic, necrosis). Indeed, in the milieu of advanced atherosclerotic lesions, phagocytosis of apoptotic cells may be compromised. For example, oxidized LDL, a CD36 ligand that exists in lesions, can competitively inhibit the phagocytic uptake of apoptotic cells.[13]

Moreover, it is theoretically possible that lipid-loaded macrophages are defective in phagocytosis due to cholesterol-induced changes in membrane structure and function or in phagocytosis-related signaling pathways. Pathological studies of advanced atherosclerotic lesions have revealed a strong correlation between macrophage apoptosis and large necrotic cores on the one hand and the incidence of plaque rupture and acute vascular events on the other.[14] While these studies are consistent with a causal relationship, definitive proof must await animal studies in which lesional macrophage apoptosis is altered by genetic manipulation or drugs. Nonetheless, mechanistic considerations suggest that macrophage apoptosis may, indeed, promote late lesional events. As alluded to above, dead macrophages release proteases that could degrade plaque-stabilizing collagen (i.e., thinning of the fibrous cap), and plaques would be expected to become friable as a result of the replacement of intact cells by cellular debris and extracellular lipid. It is also possible that the many needle-shaped cholesterol crystals in the core, which likely arise from the release of macrophage cholesterol, physically damage the thinning plaque. Finally, the fatal post-rupture event is acute thrombosis, and macrophage death may release pro-thrombotic materials, notably tissue factor.[12]

Free Cholesterol-induced Macrophage Death as a Model of Macrophage Apoptosis in Advanced Atherosclerotic Lesions

While there are a number of possible causes of macrophage death in advanced atherosclerotic lesions, death caused by intracellular accumulation of unesterified or 'free' cholesterol (FC) deserves consideration because advanced lesional macrophages accumulate FC, and FC accumulation is a potent inducer of apoptosis in cultured macrophages.[11] Moreover, the results of several mouse studies are consistent with a role for FC-induced macrophage death in advanced atherosclerosis (below).

Free cholesterol accumulation in late lesional macrophages

How do advanced lesional macrophages accumulate FC? When monocytes enter the subendothelial space of a developing atherosclerotic lesion and differentiate into macrophages, they internalize large quantities of retained atherogenic lipoproteins by receptor-mediated endocytosis and phagocytosis.[15] Most of the cholesterol in these internalized lipoproteins is esterified to fatty acids and is referred to as cholesteryl ester.

In hydrolase-containing acidic endosomes, presumably a type of 'late endosome,' these lipoprotein-derived cholesteryl ester molecules are hydrolyzed to FC and fatty acids by lysosomal acid lipase. By an important yet poorly characterized process, the FC is trafficked from late endosomes to peripheral cellular membranes, such as the plasma membrane, mitochondria, and ER.[11]

Despite the delivery of large amounts of cholesterol, early lesional macrophages maintain a relatively low FC content. While some of the FC is transported out of the cell through plasma membrane efflux pathways, most of the lipoprotein-derived FC is re-esterified to fatty acids in the ER by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT).[16] ACAT-derived cholesteryl esters are sequestered in numerous phospholipid-bound droplets in the cytoplasm, giving the cells a foamy appearance. Indeed, the macrophage foam cell is the hallmark of early atherosclerotic lesions.[17] As excess FC damages cells while cholesteryl esters do not (below), ACAT-mediated re-esterification is an important protective pathway in early lesional macrophages. In this context, cells have a mechanism to hydrolyze ACAT-derived cholesteryl esters via a cytoplasmic neutral cholesteryl ester hydrolase, but the FC derived from this reaction is rapidly metabolized, effluxed, or re-esterified by ACAT.[18]

As lesions become more advanced, there is a progressive decrease in lesional cholesteryl ester content and an increase in FC content both extracellularly and in cells.[19–22] Based on these observations, one can infer that something goes awry with the mechanisms that prevent FC accumulation in the macrophages of earlier lesions.

Most likely, several protective mechanisms become progressively defective as long-lived lesional macrophages

accumulate more and more lipoprotein cholesterol. A cell culture model has been developed in which FC accumulates in activated macrophages after prolonged exposure to atherogenic lipoproteins, followed by lipoprotein withdrawal. In this case, the mechanism appears to involve enhanced hydrolysis of ACAT-derived cholesteryl ester, perhaps coupled with defective esterification.[23] Consistent with this model, a recent *in vivo* study has provided evidence that FC accumulates in the ER of advanced lesional macrophages (below), suggesting a defect in ACAT-mediated cholesterol esterification and/or an activation of the neutral cholesteryl esterase reaction. That defective ACAT could cause lesional macrophages apoptosis was demonstrated in a mouse model of atherosclerosis in which macrophage ACAT was genetically eliminated. In this model, there was an accelerated accumulation of FC and an increase in apoptosis in lesional macrophages.[24] Finally, there are scenarios where one can envision defective cholesterol efflux in the milieu of the advanced atherosclerotic lesion. For example, there may be decreased expression of macrophage receptors (e.g., ABCA1 and SRB1) for HDL and other efflux-inducing particles, as well as limited accessibility of these particles to macrophages buried deep in advanced lesions.[25,26]

Cellular mechanism of FC-induced macrophage apoptosis

Cell culture models

As mentioned earlier, intracellular FC accumulation is a potent inducer of apoptosis in macrophages. To understand the mechanisms involved, investigators have turned to cell culture models. In the most widely used model, primary macrophages (e.g., mouse peritoneal macrophages) or macrophage-like cell lines (e.g., J774 or RAW macrophages) are incubated with lipoproteins as the source of cholesterol. In addition, an ACAT inhibitor is added to accelerate FC accumulation, because in the time frame of the typical experiment cholesteryl ester accumulation would predominate in the absence of the inhibitor.[27,28] Although the inclusion of the ACAT inhibitor is an *in vitro* manipulation, there is *in vivo* evidence to suggest that this is a valid model (below). In terms of the choice of lipoproteins, those internalized by the type A scavenger receptor (SRA) are ideal, because this receptor results in constitutive lipoprotein uptake, and the receptor is known be important *in vivo*.[29] In atherosclerotic lesions, one type of SRA ligand is low-density lipoprotein (LDL) that has undergone oxidative modification.[4]

Although oxidized LDL can induce apoptosis in macrophages under certain conditions, the mechanism is distinct from apoptosis induced by FC loading (see the following section). Rather, a more direct model of the FC-loaded macrophage *per se* can be achieved using human LDL that is converted to an SRA ligand by chemical acetylation (acetyl-LDL). Macrophage cholesterol loading using acetyl-LDL mimics the type of massive cholesterol accumulation that occurs *in vivo* by a variety of nonoxidized atherogenic lipoproteins.[30]

Caspase activation

When mouse peritoneal macrophages are incubated with acetyl-LDL and an ACAT inhibitor, effector caspase-dependent apoptosis occurs after the level of FC accumulation has reached a certain level.[11] Upon further incubation, the cells progressively appear necrotic (e.g., swollen organelles, disrupted cell membranes), thus mimicking the type of secondary necrosis that is thought to occur in advanced atherosclerotic lesions (above). Both the Fas and mitochondrial pathways of apoptosis are involved in this model. A role for the Fas pathway was proven by showing that FC loading led to caspase 8 activation and that apoptosis could be partially blocked by an anti-Fas ligand antibody or by using macrophages from *gld* (Fas ligand deficient) or *lpr* (Fas deficient) mice.[31] Further studies revealed that FC loading increases cell-surface expression of Fas ligand by a post-transcriptional mechanism, perhaps by increased delivery of intracellular stores of Fas ligand to the plasma membrane. The Fas ligand on these macrophages engages constitutively expressed Fas on neighboring macrophages and triggers apoptosis.[31]

After a relatively early level of FC accumulation, there is a marked decrease in the mitochondrial transmembrane potential of the macrophages, and this is followed by cytochrome *c* release and caspase 9 activation.[32] This series of events is not blocked by inhibiting the Fas pathway, indicating that a Fas-mitochondria cross-talk pathway, for example, via Bid activation, is not involved. FC loading also leads to a post-transcriptional increase in cellular and mitochondrial Bax levels,[32] but a direct causal relationship between the increase in Bax and apoptosis in these cells has not yet been established.

Caspase 9 activation and apoptosis still occur in FC-loaded Bax-deficient macrophages, but this could be related to compensatory mechanisms, for example, via Bak.[33]

Upstream signaling pathways

What are the upstream pathways whereby FC loading leads to these downstream apoptotic events? Previous work using general intracellular cholesterol trafficking inhibitors had suggested that an increase in the plasma membrane FC content was critical,[34] indicating either direct damage to the plasma membrane or induction of a plasma membrane-based signal transduction pathway. However, direct loading of the plasma membrane with excess FC was found not to induce apoptosis.[35] Moreover, specific inhibition of trafficking of lipoprotein-derived cholesterol from late endosomes to the endoplasmic reticulum (ER) blocked apoptosis, suggesting that FC accumulation in the ER membrane, not the plasma membrane, was key.[35] Cholesterol trafficking to the ER, but not to the plasma membrane, was blocked by using a low dose of an androgen-like amphipathic amine called U18666A or by conducting the experiments in macrophages that were partially deficient in the cholesterol trafficking protein *Npc1*.[35] Notably, macrophage apoptosis and lesional necrosis in advanced atherosclerotic lesions in mice were substantially decreased by blocking cholesterol trafficking to the ER *in vivo*.[36] The finding that the ER was important in FC-induced apoptosis suggested a role for an ER-based signal

transduction pathway. In this context, FC loading of macrophages was shown to be a potent inducer of the UPR,[35] an ER-based pathway consisting of upstream kinases and nucleases and downstream transcription factors.[37,38] The UPR is induced by a variety of physiologic and pathologic circumstances in which the protein synthesis function of the ER is 'stressed' by high levels of protein synthesis or improper folding of nascent proteins. The UPR functions primarily to ameliorate these stressors (e.g., by slowing translation or by inducing chaperones), but there is a branch of the UPR that triggers apoptosis if the corrective measures fail.[39–43] In the case of the FC-loaded macrophage, UPR induction was shown to be completely dependent on FC trafficking to the ER.[35] Most importantly, macrophages from gene-targeted mice that lack the UPR effector CHOP, which is involved in the apoptosis branch of the UPR, are highly resistant to FC-induced apoptosis despite the same level of FC loading as wild-type macrophages. Finally, CHOP mRNA and protein were shown to be widely expressed in the macrophages of advanced murine atherosclerotic lesions.[35]

How might FC loading trigger the UPR? A very early event in FC loading, and one completely dependent on cholesterol trafficking to the ER, was depletion of ER calcium stores.[35] Depletion of lumenal ER calcium is known to be a potent inducer of the UPR, probably because many ER chaperones that function in nascent protein folding are calcium-dependent proteins.[44] The molecular basis of FC-induced ER calcium depletion is not yet known. However, the ER is normally a cholesterol-poor, fluid membrane, and so the membrane lipid-ordering effect of cholesterol might alter the function of proteins involved in ER calcium homeostasis, such as calcium channels and pumps. In particular, malfunction of the sarco-endoplasmic reticulum ATPase (SERCA) would cause depletion of ER calcium stores. Likewise, a number of other integral ER membrane proteins may not function properly in highly ordered membrane, and the malfunction of one or more of these proteins may also contribute to ER stress and UPR induction.

In summary, our current working model of how accumulation of FC in macrophages leads to apoptosis is as follows (Figure 1): a portion of the cholesterol derived from the internalization and hydrolysis of atherogenic lipoprotein trafficks from late endosomes to the ER via a route and a mechanism that is not yet known. When the cholesterol : phospholipid ratio in the ER membrane reaches a certain level, one or more molecules involved in ER calcium homeostasis or other critical ER processes fail to function properly. This situation creates a 'stress' on the ER, perhaps related to improper protein folding, that induces the UPR. While much of the machinery of the UPR is dedicated to adaptive and repair processes, we must assume that these processes eventually fail and that apoptosis programmed by the CHOP branch of the UPR ensues. The mechanisms linking products of the CHOP pathway to the caspase pathways in FC-loaded macrophages are not yet known, although specific CHOP pathway-related apoptotic mechanisms have been elucidated in other systems.[45] The finding that a portion of macrophage apoptosis in advanced atherosclerotic lesions is dependent on cholesterol trafficking to the ER and that CHOP is expressed in advanced atherosclerotic lesions suggest that these events may occur *in vivo*.

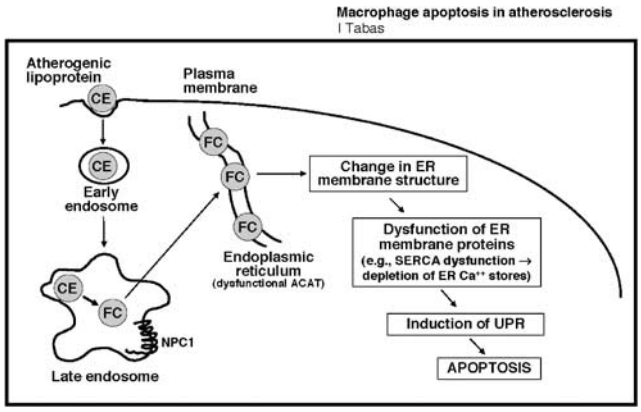

Figure 1 Model of FC-induced apoptosis in macrophages. Macrophages in atherosclerotic lesions internalize atherogenic lipoproteins, which carry most so their cholesterol as cholesteryl fatty acid esters (CE). The CE is hydrolyzed to FC in late endosomes, and a portion of the FC is trafficked to the ER. If ACAT is dysfunctional, the FC : phospholipid ratio in the ER membrane will increase, leading to stiffening and widening of the ER membrane bilayer. This change in the ER membrane, which is normally very fluid, will likely cause the malfunction of critical ER membrane proteins. One possible example is the SERCA calcium pump, the malfunction of which will lead to depletion of ER calcium stores. The consequences of ER membrane protein dysfunction, including depletion of ER calcium stores, induce the UPR. UPR induction, in turn, eventually leads to triggering apoptotic pathways via the CHOP branch of the UPR.

Oxysterol-induced Macrophage Death

Some of the lipoprotein-cholesterol that accumulates in atherosclerotic lesions is thought to be oxidized either prior to entry into the arterial wall or secondary to enzymatic or chemical processes in the lesions themselves.[4] Indeed, the ability of oxidized LDL to induce apoptosis in macrophages is thought to be mediated by oxysterols, such as 7-ketocholesterol, in oxidized LDL.[46] In terms of mechanism, Sinensky and co-workers have focused on a model system that utilizes 25-hydroxycholesterol (25-OHC) and cultured macrophages. In this system, oxysterols or relatively high doses of oxidized LDL induce macrophage apoptosis via the mitochondrial pathway.[47] The most upstream events appear to be calcium influx into the cells, cytosolic phospholipase A_2 activation, arachidonic acid release, and possibly reactive oxygen species (ROS) formation. In a recent study, this group showed that oxysterols promoted proteasome-mediated degradation of Akt (protein kinase B), a prosurvival protein, in P388D1 macrophages.[48] Presumably, this event is downstream of arachidonic acid release, ROS formation, or both, but the linking mechanism has not yet been elucidated. Later events in this model include activation of the pro-apoptotic Bcl2-family member Bad via dephosphorylation and translocation to the mitochondria, induction of pro-apoptotic Bim, and downregulation of antiapoptotic Bcl-X_L.[48] These processes would be expected to promote the mitochondrial pathway of apoptosis known to occur in oxysterol-treated cells. Paradoxically, when growth factor-dependent bone marrow-derived macrophages are exposed to relatively low doses of oxidized LDL, apoptosis induced by growth factor withdrawal

is actually prevented.[49,50] Macrophage colony-stimulating factor (M-CSF) withdrawal from these macrophages normally leads to the activation of acid sphingomyelinase, generation of ceramide, downregulation of Akt, and stimulation of the mitochondrial pathway of apoptosis.[49] When the cells are incubated with oxidized LDL, activation of acid sphingomyelinase is prevented, and cell survival is enhanced.[50]

The component of oxidized LDL responsible for this action has not yet been identified, although sphingosine-1-phosphate and ceramide-1-phosphate have similar actions.[51,52] In this model, one can presume that the level of oxysterols is not high enough to overcome the antiapoptotic effects of oxidized LDL. In summary, *in vitro* studies with oxidized LDL and oxysterols have shown both pro- and antiapoptotic effects, depending on the method of LDL oxidation, the dose of the lipoprotein, and the type of macrophage used. Whether or not oxidized LDL in atherosclerotic lesions can mediate one or both of these effects remains to be determined.

Conclusions and Future Directions

Apoptotic macrophages are present in atherosclerotic lesions, but the mechanisms and consequences of this event are not yet fully known. In terms of mechanisms, several known inducers of macrophage apoptosis are either present in lesions or could be present. This review has emphasized macrophage FC accumulation as an inducer of apoptosis, because this event does occur in advanced atherosclerotic lesions, and recent studies have provided insight into the cellular and molecular mechanisms. Most importantly, a mouse model in which a specific cellular event required for FC-induced apoptosis – trafficking of lipoprotein cholesterol to the ER – is interrupted has provided evidence that this mechanism of macrophage apoptosis is relevant to advanced atherosclerosis. Oxidized LDL and oxidized lipids are also known to occur in atherosclerotic lesions, but whether the levels are high enough to induce the pro- or antiapoptotic mechanisms described above is not yet known. Other possible inducers of macrophage apoptosis relevant to advanced lesions in particular include cytotoxic cytokines like TNF and growth factor deprivation. As with oxidized LDL, the *in vivo* relevance of these inducers remains to be determined.

The consequences of lesional macrophage apoptosis may depend on the stage of the lesion in which cell death occurs and the relative ability of phagocytes to ingest the apoptotic cells. This review has emphasized late lesional events, in which phagocytic clearance of apoptotic cells is likely suboptimal (above). In this case, apoptosis lead to secondary necrosis, and necrosis in turn almost certainly promotes plaque destabilization for the reasons outline in this review. In early lesions, however, macrophage apoptosis might also occur, and phagocytic clearance is probably less compromised in this setting. Therefore, macrophage apoptosis in early lesions may result in a decrease in lesional cellularity, which would be expected to be antiatherogenic. Therefore, a critical area of study for future investigation is to elucidate the stage-specific consequences of macrophage apoptosis in the development and progression of atherosclerotic lesions. Such studies may lead to the development of novel therapeutic strategies that target atherogenesis and plaque destabilization.

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