Cell Death in the Vessel Wall
The Good, the Bad, the Ugly

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Atherosclerotic vascular disease results from an imbalance of inflammatory and vascular cell accumulation and removal in the neointimal space. When pathways that promote cell recruitment, survival, and proliferation are favored over those that activate cell death, egress, and clearance, the plaque expands. In contrast, programmed cell death and the efficient clearance of apoptotic bodies by efferocytosis reduce lesion cellularity and promote a reparative environment and lesion stability. However, should these carefully balanced pathways become disturbed, lesions can accumulate cell debris, damaged associated molecular patterns, and arrested macrophages, all contributing to the proinflammatory environment and lesion instability. Here, we review the latest understanding of how cell death in the vessel wall directly coordinates the development of atherosclerosis, and what molecular signals are orchestrating these pathways. We discuss the necessity of cell death and the ways in which the execution of different forms of cell death can direct different outcomes in the plaque, and how promoting the effective clearance of dead cells from the lesion is looking like a promising therapeutic path forward.

In the Beginning
In the setting of excess cholesterol and fat, inflammatory pathways of the innate immune system are aberrantly activated. It is widely believed that oxidative modifications of the phospholipid and protein moieties of low- and very–low-density lipoproteins trigger conserved nonspecific scavenging pathways in macrophages and dendritic cells. This initiates what is at first considered a protective pathway to remove unwanted and potentially cytotoxic remnants and debris. However, during the decades of life wherein circulating low- and very–low-density lipoprotein cholesterol are in excess, this protective response turns into a chronic state of inflammation that recruits additional inflammatory cells and activates vascular cells to promote lesion expansion.

During the progression of atherosclerosis, like in many other diseases, there is a constant turnover of cells within and surrounding the plaque. The identification in recent years of the key factors that mediate cell recruitment, proliferation, death, and migration out of the plaque has led to an expansion in the understanding of how vascular lesions develop.1

With this has come an appreciation of the importance of cell death in maintaining a healthy neointimal space. Indeed, early histopathologic studies in humans and mice showed that macrophages, smooth muscle cells (SMC), and endothelial cells all undergo apoptosis, though whether this was a protective or disease-causing mechanism was debated.2 In the past decade, direct evidence and mechanistic insight into how cell death contributes to atherosclerosis at all stages has been better elucidated, and we focus on recent evidence connecting the drivers of apoptosis, necrosis, and efferocytosis to the progression and advancement of atherosclerotic disease.

The Good
Apoptosis in Early Lesions
Early work by Liu et al3 was the first to show that macrophage apoptosis is necessary to reduce macrophage burden within the developing lesion in Ldlr−/− mice. While initially apoptosis was thought to directly contribute to necrotic core expansion and, thus, reducing apoptosis might lead to reduced atherosclerosis, reconstituting the bone marrow compartment of Ldlr−/− mice with hematopoietic cells lacking the proapoptotic factor Bax led to accelerated lesion development. Similarly, the antiapoptotic protein AIM (apoptosis inhibitor of macrophages) promotes macrophage survival and, when deleted, accelerates macrophage apoptosis and reduces lesion size in AIM−/−Ldlr−/− mice.4 Together, this evidence confirmed that lesional macrophage apoptosis is necessary, at least initially, to reduce the pool of macrophages within the expanding lesions.

We now have a better understanding of the signals that promote apoptosis in early atherosclerotic lesions and whose loss may be driving inflammatory lesion development. In response to cholesterol accumulation and endoplasmic reticulum stress, macrophages activate the unfolded protein response and undergo apoptosis.5–7 Within endoplasmic reticulum–stressed macrophages, the activation of the nuclear factor-kB signaling cascade by IKKα (IκB kinase) can promote a prosurvival pathway, and deletion of IKKα in macrophages in Ldlr−/− mice leads to increased early lesional apoptosis and reduced overall atherosclerotic lesion burden.8 In contrast, the proapoptotic factors Jnk1 and Jnk2 (c-Jun N-terminal kinase 1 and 2) are activated by multiple stress pathways, and their activity is balanced by the prosurvival factors PI3K and Akt.9 In mice, deletion of Jnk1 in hematopoietic cells increased lesion area in Ldlr−/− mice, whereas deletion of Jnk2 had no impact on lesion size.10 In macrophages, Jnk1 but not Jnk2 controls the activity of Akt, so in the absence of Jnk1, there is enhanced prosurvival Akt signaling, leading to macrophage expansion within the lesion. Together, these studies suggest that in macrophages, there is a careful balance between inflammatory and prosurvival pathways that, when activated in favor of proapoptotic pathways,
contribute directly to macrophage accumulation and inflammation in the vessel wall (Figure).

**Efferocytosis: Apoptosis’ Partner in Crime**

In healthy tissues, apoptotic cells are cleared rapidly by local phagocytes in a process known as efferocytosis. In fact, apoptotic bodies are cleared so rapidly in vivo that they can be difficult to detect using traditional histological methods. During the nascent stages of atherosclerosis, efferocytosis of apoptotic foam cells is mediated largely by the receptor MerTK (tyrosine-protein kinase Mer), and mutations or deletion of MerTK leads to a dramatic increase in apoptotic cell accumulation within lesions and an increase in lesion size and necrotic core. These studies positioned MerTK as critical for the protection from atherosclerosis via efficient clearance of apoptotic bodies. Recently, additional mechanisms for efferocytosis control have been uncovered using both human and mouse genetics. ADAM17 (ADAM metallopeptidase domain 17) is a metalloproteinase that was identified as a candidate atherosclerotic gene by quantitative trait mapping in mice and has been found to be elevated in human plaques that had undergone plaque rupture. MerTK has been shown to be proteolytically cleaved by ADAM17, and cleavage of MerTK promotes necrotic core expansion and increased atherosclerotic lesion size, presumably because of its inability to effectively clear apoptotic bodies. Therefore, it was assumed that elevated levels of ADAM17 in the plaque would result in accumulation of apoptotic debris and expansion of atherosclerotic lesions. However, recently, Nicolaou et al showed that in Ldlr−/− mice with a hypomorphic mutation that lowers ADAM17 expression, ADAM17-deficient cells had reduced apoptosis, and atherosclerotic lesions from these mice had increased numbers of both macrophages and SMCs. This was attributed to the increased signaling through tumor necrosis factor receptor 2 and membrane-bound tumor necrosis factor-α, which promoted cell survival and proinflammatory signaling. Although in this study, the function of ADAM17 deficiency on efferocytosis and MerTK cleavage was not assessed, clearly the multifactorial role for ADAM17

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**Figure.** Cell death during early and advanced atherosclerosis. In early atherosclerosis (top), lesion cellularity is limited via apoptosis through activation of endoplasmic reticulum (ER) stress and proapoptotic factors (e.g., Jnk1 [c-Jun N-terminal kinase 1], Bax-1 [Bcl-2–associated X protein]) or through downregulation of antiapoptotic factors (Bcl-2 [B-cell lymphoma 2], AIM [apoptosis inhibitor of macrophages], IKKα [IκB kinase]). Efficient efferocytosis via MerTK (tyrosine-protein kinase Mer) and adequate Resolvin synthesis clears apoptotic body accumulation. Other anti-inflammatory and antiapoptotic factors (e.g., ADAM17 [ADAM metallopeptidase domain 17] and TNFR2 [tumor necrosis factor receptor 2]) also reduce lesion expansion. In advanced lesions (bottom), excessive apoptosis and activation of necroptosis (e.g., RIP3 [receptor-interacting protein kinase 3], MLKL [mixed lineage-kinase domain-like]) can cause release of necrotic material that may be recognized by receptors like Ced4e and activate further apoptosis or inflammation. Don’t eat me signals presented on dying atherosclerotic cells prevent efferocytosis clearance. Defective efferocytosis via cleavage of MerTK, reduced Resolvin synthesis, or miR-155-dependent inhibition of Bcl6 (B-cell lymphoma 6) also prevent clearance of apoptotic and necrotic cells. Cell debris accumulates and necrotic core expands in advanced atherosclerosis.
in atherosclerosis in mice and humans warrants further investigation.

Large genome-wide association studies have revealed novel genomic regions that associate strongly with the risk of coronary artery disease, yet, for which no known mechanism exists. For instance, the Tim (T cell immunoglobulin and mucin domain) proteins have genetic associations with serum triglyceride levels and risk for coronary artery disease. Because they are expressed by immune cells and have been shown to mediate apoptotic cell clearance, Foks et al hypothesized that Tim proteins could play a role in the pathogenesis of atherosclerosis. Treatment of Ldlr−/− mice with blocking antibodies directed against Tim-1 or Tim-4 significantly enhanced atherosclerosis compared with control IgG-treated animals, largely by preventing the clearance of CD4+ T cells and apoptotic macrophages. Another strong genetic risk locus for CAD is found on chromosome 9p21, which contains large stretches of noncoding and coding regions, some of which have been found to mechanistically contribute to atherosclerosis. Kojima et al found that this locus regulates the expression of the proinflammatory molecule calreticulin, and impaired expression of calreticulin reduces efferocytosis and accelerates atherosclerosis. This prompted the group to further investigate mechanisms of impaired efferocytosis in the vessel wall and found that the don’t-eat-me signal CD47 was paradoxically upregulated in SMC foam cells, and blocking CD47 by several approaches resolved atherosclerosis via improving efferocytosis.

Defective Efferocytosis

Despite the accumulation of free cholesterol and the detectable activation of the unfolded protein response in macrophages in early lesions, efferocytosis occurs so rapidly that apoptotic cells are difficult to find. However, as lesions progress, the accumulation of apoptotic cells becomes apparent in the advanced plaque. The hypothesis that defective efferocytosis contributed to this was first confirmed by Schrijvers et al who found direct evidence of impaired phagocytosis of apoptotic cells in human and rabbit arteries. Subsequently, this hypothesis was solidified by various studies demonstrating that rendering efferocytosis defective can directly contribute to advanced lesion development.

But what are the mechanisms by which efferocytosis can become defective? One mechanism recently identified involves the resolvin family of lipid mediators. During the inflammatory response, fatty acids eicosapentaenoic acid and docosahexaenoic acid are processed by the lipoxygenase enzymes 5-LOX and 12/15-LOX, respectively, to generate the resolvins E and D (RvE and RvD). Resolvins serve to limit inflammation via blocking the production of cytokines and limiting neutrophil infiltration but, importantly, also promote efferocytosis during the resolution of inflammation. To test the contribution of resolvins to the development of atherosclerosis, Hasturk et al administered RvE1 to rabbits while simultaneously feeding a high-cholesterol diet and found a significant attenuation of plaque formation, inflammation, and necrotic core area in RvE1-treated animals. Although efferocytosis was not directly evaluated in this study, the premise that resolvins could serve to limit lesion progression was established. Indeed, a defect in resolvin synthesis was found in advanced human plaques, and replacement of RvD1 in established lesions in mice reduced necrotic core size by enhancing lesional efferocytosis.

Other proresolving mediators RvD2 and Maresin-1 prevented...
atherosclerosis in mice when delivered in conjunction with a high-fat diet, restoring these mediators to levels seen in stable plaques. These studies provide compelling evidence that defective efferocytosis is a consequence of defective production of proresolving lipid mediators and can be used as an intervention in established atherosclerosis.

Additional pathways may also underlie defective efferocytosis in the advanced atherosclerotic plaque. For example, Annexin A1 is a ligand that is important for the clearance of apoptotic cells. De Jong et al recently found that Annexin A1 expression in the vessel wall and in the circulation negatively correlated with neointima size, suggesting that it may serve to protect from lesion development by enhancing efferocytosis. In addition, the endogenous expression of miR-155 is found to be elevated during the progression of atherosclerosis in mice coincident with the downregulation of Bcl6 and defective efferocytosis. Accordingly, when miR-155 is blocked, efferocytosis is restored. Together, these mechanisms offer multiple possible ways by which efferocytosis becomes defective as lesions progress, and further studies are needed to understand how these factors become dysregulated in the plaque.

The Ugly

Pyroptosis and Necroptosis

In addition to apoptosis, other emerging forms of programmed cell death have been found to be active in the vessel wall and in some cases contribute to the progression of atherosclerosis. Among the most well-defined are pyroptosis and necroptosis. Pyroptosis is a caspase-dependent form of lytic cell death that shares features with both apoptosis and necrosis. When the inflammasome is triggered via innate immune toll-like and nod-like receptors, this induces the aggregation and activation of the multi-protein inflammasome complex which, in atherosclerotic macrophages, includes NLRP3 (NLR family pyrin domain-containing 3), ASC (apoptosis-associated speck-like protein containing a CARD), and caspase-1. Caspase-1 cleaves pro-interleukin-1β into its mature form for secretion, but in some cases can form a pore in the plasma membrane, allowing release of cellular contents and cell death. Whether caspase-1 induces inflammation or cell death depends critically on the levels of caspase-1 in the cell. In atherosclerotic plaques that have undergone rupture, dead or dying macrophages have greater caspase-1 expression than what is seen in stable plaques. Recently, Emrich et al found that SMC apoptosis and caspase-1 activity were elevated in aortic aneurysm disease in a mouse model of Marfan’s syndrome, and treatment of these mice with an inhibitor of caspases reduced SMC apoptosis and overall disease burden. Similarly, excess circulating cholesterol induces the activation of caspase-1 in the endothelium of ApoE−/− mice, and in vitro treatment of endothelial cells with oxidized lipids induces pyroptosis. Endothelial activation, inflammation, and early atherosclerosis in ApoE−/− mice is subsequently inhibited by the deletion of caspase-1, which confirms earlier reports that caspase-1 plays a key role in vascular disease progression. In human macrophages, p38β-mitogen-activated protein kinase is necessary for the activation of caspase-1 via the NLRP3 inflammasome and is upregulated in advanced but not early human atherosclerotic plaques. Although many of these studies do not dissect the precise contribution of the pro-death versus proinflammatory roles for caspase-1, they underscore the potential importance of caspase-1 regulation as a key determining factor in the inflammation that drives atherosclerosis.

Programmed necrosis, or necroptosis, is a more recently defined form of programmed cell death. Necroptosis differs from both apoptosis and pyroptosis mainly because it is not dependent on caspase activation. When a death receptor is triggered, RIP (receptor-interacting protein) kinase 1 becomes activated together with other death effector proteins TRADD (tumor necrosis factor receptor type 1-associated DEATH domain), FADD (Fas-associated protein with death domain), and caspase-8 to promote apoptosis. When caspase-8 is inactive or overwhelmed, RIP1 interacts with and phosphorylates RIP kinase 3 (RIP3) to form the oligomeric necrosome. This phospho-RIP1/phospho-RIP3 complex then recruits and phosphorylates the multilineage kinase-like domain MLKL (mixed lineage-kinase domain-like), which further oligmerizes and facilitates membrane rupture via pore formation and ion release. The result is a cell that shares morphological features with necrosis and the release of ATP, mitochondria, and other damage-associated molecular patterns. Until recently, it was thought that the necrosis observed in advanced atherosclerotic lesions, particularly in the large necrotic cores found in ruptured human plaques, was a consequence of secondary necrosis because of failed efferocytosis. Lin et al demonstrated that RIP3 could directly contribute to atherogenesis and that deletion of RIP3 in either Ldlr−/− or ApoE−/− mice reduced necrotic core size. Our group demonstrated that in the setting of established atherosclerosis in ApoE−/− mice, inhibition of the RIP1 activation of RIP3 via the small molecule Necrostatin-1 reduced lesion size and, importantly, necrotic core formation. We also found evidence of active necroptosis in advanced but not early coronary atherosclerotic lesions in humans, suggesting that this process may be contributing to human disease. Although the activators of active necroptosis in the lesion are continuing to be established, it is conceivable that pathways that exacerbate or protect from necrosis in advanced lesions could be doing so through a necroptosis mechanisms. For example, activation of the necrosis-sensing receptor Cecl4e in atherosclerotic plaques, which has been shown to propagate atherosclerosis via the endoplasmic reticulum–stress response, could be achieved through the release of damage-associated molecular patterns from a necrotic cell. Interestingly, atherosclerotic ligands can activate the glycolytic pathway in macrophages via the glucose transporter PFKFB3 (6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase 3), which potentiates their inflammatory activation. Blocking PFKFB3 accelerates the rates of apoptotic and necrotic death in macrophages, possibly because of the high glycolytic rate needed to maintain their viability. Although RIP kinases and necroptosis were not directly evaluated in this study, it is plausible that the balance of apoptosis and necroptosis in atherogenic macrophages is carefully controlled by the energetic needs of the cell.

Emerging Concepts

In more recent years, our global understanding of how atherosclerotic lesions develop and progress has changed. In particular, the origin of the macrophage foam cells found within advanced lesions has been challenged and has now
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