Cell Death, Damage-Associated Molecular Patterns, and Sterile Inflammation in Cardiovascular Disease

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Abstract—Cell death and inflammation are ancient processes of fundamental biological importance in both normal physiology and pathology. This is evidenced by the profound conservation of mediators, with ancestral homologues identified from plants to humans, and the number of diseases driven by aberrant control of either process. Apoptosis is the most well-studied cell death, but many forms exist, including autophagy, necrosis, pyroptosis, paraptosis, and the obscure dark cell death. Cell death occurs throughout the cardiovascular system, from initial shaping of the heart and vasculature during development to involvement in pathologies, including atherosclerosis, aneurysm, cardiomyopathy, restenosis, and vascular graft rejection. However, determining whether cell death primarily drives pathology or is a secondary bystander effect is difficult. Inflammation, the primary response of innate immunity, is considered essential in initiating and driving vascular diseases. Cell death and inflammation are inextricably linked with their effectors modulating the other process. Indeed, an evolutionary link between cell death and inflammation occurs at caspase-1 (which activates interleukin-1β), which can induce death by pyroptosis, and is a member of the caspase family vital for apoptosis. This review examines cell death in vascular disease, how it can induce inflammation, and finally the emergence of inflammasomes in vascular pathology. (Arterioscler Thromb Vasc Biol. 2011;31:2781-2786.)

Key Words: apoptosis ■ atherosclerosis ■ immune system ■ macrophages ■ vascular biology

Unraveling the contribution of cell death, and in particular apoptosis, to multifactorial vascular diseases is complex, and in many cases contradictory. Cell loss has direct effects stemming from its absence, and thus inability to undertake its function (eg, endothelial cell barrier function), and secondly the tissue’s response to cell loss (eg, fibrotic scar formation following myocyte loss). Core factors of the apoptotic machinery are ubiquitous and highly conserved, making pharmacological manipulation problematic. However, a number of studies have been performed.

For example, antisense inhibition of the anti-apoptotic Bcl-XL in rabbit carotid arteries reduced neointimal hyperplasia after injury,1 whereas reduced plaque apoptosis in fat-fed p19ARF−/−/apolipoprotein E (ApoE)−/− mice increased aortic arch plaque, with no effect on plaque composition.2 These studies suggest that apoptosis in multiple cell types reduces atherosclerosis. In contrast, adenoviral expression of pro-apoptotic Fas ligand in rabbit carotids accelerated lesion growth with more vascular smooth muscle cells (VSMCs),3 whereas p53−/−/ApoE−/− mice (p53 induces apoptosis) had reduced macrophage apoptosis and increased lesion area but demonstrated a protective role for p53 in VSMCs.4 Thus, the true effects of apoptosis at given stages of vascular pathologies can be understood only by using cell type–specific interventions, ideally with conditional systems. The 2 main approaches are manipulation of factors that either kill or protect a cell, or manipulation of phagocytic machinery that clears dead cells.

Manipulation of VSMC Death

Previous studies have shown that induction of apoptosis in ≤10% of cells in a reseeded rat carotid artery neointima increased monocyte chemotactic protein-1 and interleukin (IL)-8, leading to large influxes of macrophages, which was partially IL-1α dependent.5 As translation is inhibited during apoptosis,6 this data indicated that dead cells induce viable cells to produce proinflammatory cytokines, and that VSMC death promoted inflammation. However, the effects of apoptosis appear to depend on the surrounding extracellular milieu. For example, inducing apoptosis in up to 70% of VSMCs was silent in SM22α-hDTR mice (which express the human diphtheria toxin receptor in VSMCs), with no inflammatory infiltrate, aneurysm formation, compensatory proliferation, or change in vessel mechanical properties.7 In contrast, inducing VSMC apoptosis within plaques of SM22α-hDTR/ApoE−/− mice caused fibrous cap thinning, collagen/matrix loss, cell debris accumulation, foci of macrophages, and chronically increased monocyte chemotactic protein-1 and IL-6.7 Although
high levels of diphtheria toxin–induced apoptosis were unphysiological, this model reinforced the idea that VSMCs stabilize plaques. This concept is supported in 2 in vivo systems that protect VSMCs against apoptosis: hepatic-expressed soluble N-cadherin and an Akt+/−/ApoE−/− model that removes a fundamental VSMC survival pathway, thus increasing apoptosis. Modulation of VSMC survival altered fibrous cap size, matrix composition, and necrotic core size, but neither manipulation changed plaque size. We have also shown that low-level VSMC apoptosis (≤1%; a level seen in human plaques) throughout atherogenesis or during progression of established plaques increased atherosclerosis =2-fold, increased proinflammatory cytokines, and still led to vulnerable plaques, along with other complications, such as calcification, elastic lamina breakdown, and medial degeneration.

**Manipulation of Macrophage Death**

Macrophages are the first cells detected subendothelially; fatty steaks are composed almost entirely of them and they are key to atherogenesis. At all stages of atherosclerosis, macrophages are the principal cell type dying, suggesting that the importance of cell death within plaques should be revealed by modulating macrophage killing. However, the published studies are not consistent. For example, adoptively transferring p53−/− marrow into ApoE−/− mice and fat feeding for 12 weeks generates plaques with less apoptosis, more macrophages, and necrotic core and doubling of lesion area. Diphtheria toxin administration to ApoE−/− mice expressing CD11b-hDTR shows that killing macrophages during plaque genesis (0 to 12 weeks of fat feeding) retards plaque growth, consistent with macrophages promoting atherosclerosis, although macrophage apoptosis in established plaques (12 to 22 weeks) reduces macrophages but does not affect plaque composition, inflammation, or size. Similarly, deletion of phospholipase Cβ3 causes macrophage hypersensitivity to apoptosis, resulting in fewer macrophages and smaller lesion size, whereas genetic deletion of antiapoptotic Bcl-2 in macrophages increases apoptosis but does not change lesion size. These experiments show that apoptosis determines macrophage content of plaques but either has no effect or reduces plaque size.

In contrast, other studies suggest that macrophage apoptosis promotes necrotic core formation and atherosclerosis. C/EBP homologous protein−/− mice have reduced macrophage apoptosis in response to cholesterol-induced endothelial dysfunction but still have reduced apoptosis, necrotic core, and plaque in both ApoE−/− and low-density lipoprotein receptor−/− backgrounds, suggesting that macrophage apoptosis increases plaque progression. Finally, an elegant study from Gautier et al examined both protection from and induction of apoptosis in early (5 weeks) and mature (15 weeks) plaques. Expressing Bcl-2 in macrophages increased lesion size during atherogenesis but ultimately retarded progression to established plaques. Inducing apoptosis using CD11c-hDTR in mature plaques resulted in modest size increases in aortic roots but larger increases in the aorta. Interestingly, recruitment of monocytes occurred in response to macrophage death, which likely resulted from increased chemokines and adhesion molecules. Again, this reinforces the idea that macrophages are essential for initiating plaque formation but that apoptosis has divergent effects depending on plaque maturity.

Clearly the outcome of cell death is dependent on the model used (protection or induction), lesion stage, cell type examined, duration of induction, etc. From a logical point of view, if cells within vascular lesions died in a truly silent manner—such that they effectively disappeared—lesions would theoretically regress. That they do not in most models indicates that the true consequences of cell death lie in the downstream response, which may accelerate lesion progression, rather than cell death per se.

**How Does Cell Death Lead to Inflammation?**

The immune system can differentiate between forms of cell death and therefore responds accordingly to perceived threat based on this. Thus, apoptosis within normal physiology is generally silent, with infected or compromised cells removed in a swift, efficient manner without immune activation. Indeed, many studies demonstrate a direct antiinflammatory and tolerizing effect of apoptotic cells. However, necrosis, which generally occurs because of physical trauma, infection, or toxic insult, activates innate immunity and mobilizes cells and mediators able to remove the stimuli and initiate tissue repair. Necrotic cell death occurs in many vascular diseases, and emerging evidence suggests that this may be a key trigger that activates the innate and adaptive immunity that drives many pathologies.

**Necrotic Cells Can Activate Innate Immunity**

Innate immunity is a rapid, first-line response toward non-self or altered-self. Molecular motifs, common to microorganisms but absent in mammalian cells, known as pathogen-associated molecular patterns act as specific ligands to elicit 2 complementary defense strategies. Binding of pathogen-associated molecular patterns to pattern recognition receptors on the cell surface, primarily Toll-like receptors (TLRs), leads to signaling via nuclear factor-κB and mitogen-activated protein kinase pathways resulting in proinflammatory cytokine expression (IL-1, tumor necrosis factor-α, IL-12), chemokine secretion (IL-8, monocyte chemotactic protein-1), and thus leukocyte infiltration and inflammation; alternatively, pathogen-associated molecular patterns recognized by scavenger receptors (eg, CD36) are endocytosed, and the microbe is digested. Infection clearance by innate immunity is less specific but fast, and collateral local tissue damage may occur. However, induction of inflammation does not have to involve microbes. Ischemia, mechanical trauma, and transplantation can cause inflammation without infection, which lead to the concept that immune systems evolved not only to respond to infections, but also to nonphysiological cell death, danger and stress. This danger hypothesis indicates that endogenous self-adjuvants released during cell death could induce inflammation without infection, and were termed damage-associated molecular patterns (DAMPs). Indeed, this sterile inflammation in response to DAMPs is key to the pathogenesis of gout, atherosclerosis, ischemia-reperfusion, and Alzheimer disease.
Sterile Inflammation in Vascular Disease

In atherosclerosis, 3 reported DAMPs trigger proinflammatory responses: cholesterol crystals, oxidation-specific epitopes, and IL-1α. Large cholesterol crystals are a feature of advanced atherosclerosis, but recently it was shown that small crystals appear after only 2 weeks of atherogenic diet in ApoE−/− mice. Cholesterol crystals deposited subendothelially activate NOD-like receptor family, pyrin domain containing-3 inflammasome, inducing IL-1β secretion21,22 (see below). This local sterile inflammation correlated with the earliest immune cell recruitment, indicating the importance of DAMPs in initiating atherogenesis. Oxidation-specific epitopes, such as oxidized phospholipids, are present on both oxidized low-density lipoprotein and apoptotic bodies, which can lead to competition for binding to scavenger receptors and may cause secondary necrosis due to failed phagocytosis. Oxidation-specific epitopes may also activate TLRs, generating inflammation. For example, activation of TLR4 on endothelial cells by oxidized 1-palmitoyl-2- arachidonoyl-sn-glycero-3-phosphocholine initiates secretion of proinflammatory cytokines and chemokines, and adhesion molecule expression, leading to monocyte recruitment. Oxidized low-density lipoprotein may also be considered a DAMP given its role in creating a local inflammatory environment within early atherosclerosis, although lipopolysaccharide contamination of many oxidized phospholipid reagents may be responsible for the TLR-activation reported.23

In contrast, IL-1α is likely involved in mature plaques when more VSMC death occurs and thus may be detrimental to plaque stability. VSMC death primarily occurs by apoptosis, but under conditions of impaired phagocytosis within advanced plaques secondary necrosis likely also occurs.22 Necrotic VSMCs release IL-1α, which activates adjacent viable VSMCs to produce the proinflammatory cytokines IL-6 and monocyte chemotactic protein-1.24 Low-level VSMC death could sustain local inflammation in advanced plaques, with activated macrophages inducing more VSMC death,25 and release of matrix-metalloproteinases that weaken the plaque further. Arteriosclerosis is the main contributor to graft failure after transplantation, and although adaptive immunity is important, the trigger for such responses was unknown. Rao et al demonstrated that IL-1α from necrotic endothelial cells promotes graft arteriosclerosis via activation of memory CD4+ T cells.26 Necrosis-derived IL-1α induced memory T-cell proliferation and secretion of IL-17 and interferon-γ, which both drive graft arteriosclerosis. IL-1α’s triggering of adaptive responses in this model may hint that DAMPs can act as tipping points from innate to adaptive immunity postinjury. Thus, IL-1α is an important DAMP associated with the vasculature. Indeed, knockout of the IL-1 receptor-antagonist, which antagonizes IL-1, results in unopposed IL-1 activity and a lethal arterial inflammation with massive migration of macrophages, neutrophils, and T cells, eventually leading to vessel wall degeneration, infarction, and aneurysm.27

Other work suggests that IL-1α is not a true DAMP but rather a secondary signal released from necrosis-sensing cells to trigger sterile inflammation in vitro and in vivo. In this...
model, primary DAMPs, such as monosodium urate, stimulate local immune cells, which in turn secrete IL-1α. IL-1α subsequently induces expression and release of chemokines to cause infiltration. These observations are in contrast with other models and prompt the question of why some necrotic cells do not use IL-1α as a direct DAMP, despite containing ample quantities. A multitude of potential DAMPs are released on necrosis, many of which require receptors with limited expression patterns. Therefore, depending on the local environment where necrosis occurs, necrotic cells may not leak the correct or sufficient DAMPs for surrounding cells to respond. To initiate a response in such settings a tissue resident sentinel immune cell could sense necrosis and release IL-1α to activate local cells as an amplification and thus generate sufficient inflammatory signals that it alone could not. Indeed, IL-1R1 appears to be widely expressed on nonimmune cells, and therefore IL-1α is perhaps a universal DAMP able to amplify or directly signal necrosis to multiple cell types.

The Role of Inflammasomes in Cardiovascular Disease

IL-1β is a potent proinflammatory cytokine produced mainly by macrophages that has key effects in vascular disease. It causes upregulation of adhesion molecules on endothelial cells, which recruits immune cells, and induces a panel of additional proinflammatory mediators. IL-1β is activated by inflammasomes—molecular platforms that in response to proinflammatory stimuli assemble multiple factors to induce activation of caspase-1, which in turn activates IL-1β, IL-18, or both, leading to secretion. IL-1β and IL-18 exist as inactive precursors and do not contain classic secretory signal sequences, and thus regulation of both processing and release provides dual control. The complexity of inflammasome assembly reflects the importance of controlling a system that leads to large-scale proinflammatory cytokine release with subsequent immune cell recruitment and activation, which often leads to collateral tissue damage.

Inflammasomes comprise self-oligomerizing scaffold proteins usually from the nucleotide-binding oligomerization domain-like receptor (NLR) family, and accessory proteins. There are 22 NLR-encoding genes in humans (and more in mice) characterized by 3 common domains: C-terminal leucine-rich repeats, central nucleotide-binding oligomerization (NACHT) domain, and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. Leucine-rich repeats are responsible for ligand recognition and autoinhibition, NACHT domains use ATP to activate the signaling complex, and CARD/PYD domains mediate homotypic protein-protein interactions. Four inflammasomes have been identified to date, based on NLRP1, NLRP3, NLRC4, and the non-NLR absent in melanoma-2 (Figure 2).

The NLRP3 inflammasome is most well understood and consists of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) adaptor and caspase-1, and it is activated by a range of microbial and endogenous signals. When NLRP3 leucine-rich repeats detect ligand the NACHT domain causes oligomerization and PYD clustering and interaction with ASC PYD domains. ASC CARD domains then interact with procaspase-1 CARDs, which autocleave caspase-1 into the active p10/p20 tetramer, allowing processing and activation of cytokines. NLRC4 has an extra C-terminal CARD that directly interacts with procaspase-1, meaning that ASC increases inflammasome efficiency but is unnecessary. NLRC4 also uses ASC to maximize caspase-1 activation. Absent in melanoma-2 is specific for double-stranded DNA recognition and not being a NLR complex it oligomerizes using multiple HIN domain binding sites on the ligand. ASC and caspase-1 are also members of this complex, and interactions are similar to those with NLRP3.

The innate immune system is a highly integrated system and inflammasome activity is integrated with TLR ligation. Priming through TLRs (eg, by lipopolysaccharide) leads to pro-IL-1β upregulation and nuclear factor-κB-dependent NLRP3 induction necessary for inflammasome function. Several potential inhibitors are present within cells, such as caspase-12, CARD, or PYD-containing proteins that sequester ASC or caspase-1, and antiapoptotic proteins (Bcl-2 and Bcl-XL) that inhibit ATP binding to NACHT. Because disparate stimuli activate inflammasomes, a direct ligand interaction may not always occur, suggesting an upstream pathway that amalgamates these signals is present. Three pathways have been proposed: reactive oxygen species produced by activators may bind NLRP3 directly or modify/bind an adaptor; a proteolytic cascade may be triggered by lysosomal damage; or a potassium efflux-triggered change in polarization may cause cellular stress.

Inflammasome Activation Can Drive Cardiovascular Disease

Inflammasome activation occurs in pathogen responses and autoimmune disorders and has recently been implicated in...
chronic conditions such as atherosclerosis and reperfusion injury. During atherogenesis, chronic inflammation and infiltration of monocytes lead to accumulation of macrophages in the developing plaque. IL-1β expression correlates with disease severity, and as a proximal mediator of the inflammatory cascade, it induces secretion of other proinflammatory cytokines and chemokines and endothelial expression of adhesion molecules and nitric oxide synthase. A stimulus suggested for this process is phagocytosis of cholesterol crystals, which triggers release of IL-1β from macrophages. Cholesterol within necrotic cores was previously considered an effect rather than a cause of lesion development, but recent work reports that minute crystals within very early plaques cause phagolysosomal membrane rupture after ingestion, leading to NLRP3 inflammasome activation, IL-1β release, and advancement of atherosclerosis. However, another study found that although cholesterol crystals induced IL-1β release, no difference in atherosclerosis occurred when mice without inflammasome components were crossed onto ApoE -/- mice. Lastly, recent work has demonstrated that basic calcium phosphate crystals also activate NLRP3 inflammasomes, suggesting that calcification may also serve as an important DAMP in vascular disease.

Revascularization after myocardial infarction triggers a massive inflammation that contributes up to 50% of the infarct area. Three hours after reperfusion injury, there is myocardial damage and hemorrhage due to reactive oxygen species production and potassium efflux, which activates inflammasomes in cardiac fibroblasts, leading to peak IL-1β and IL-18 release. From 6 to 48 hours, subsequent recruitment of macrophages and neutrophils with high ASC expression indicates further inflammasome involvement, and ASC -/- mice have smaller infarct areas and decreased macrophage and neutrophil infiltration into the ischemic myocardium, compared with wild-type mice.

**Inflammasomes Can Induce Cell Death, and Cell Death Can Activate Inflammasomes**

Pyroptosis is a catastrophic form of cell death most commonly found in monocytes, macrophages, and dendritic cells. It has biochemical and morphological characteristics of necrosis and apoptosis, but active release of IL-1β and IL-18 also occurs. Cell lysis arises because of caspase-1-dependent pore formation in the plasma membrane, which allows potassium efflux, osmotic pressure, water influx, and swelling. Inflammasomes are activated, proinflammatory proteins are released, DNA is damaged, metabolic enzymes are cleaved, and cellular disruption releases other DAMPs. Caspase-1 has a biphasic effect, where low levels cause survival responses and cytokine production, whereas levels above a threshold lead to pyroptosis. Indeed, caspase-1 may have a role in macrophage death at sites of plaque rupture in sudden coronary death. Dying macrophages within ruptured plaques have high levels of caspase-1 and little caspase-3, compared with stable plaques and control tissue. Finally, during necrosis, the NLRP3 inflammasome can be activated. Release of viable mitochondria into the extracellular space results in discharge of ATP, which acts as a powerful DAMP, explaining why some methods of cellular injury induce inflammasome activation, with pressure, complement lysis, and hypoxia allowing ATP release, whereas freeze-thaw or UV does not.

**Summary**

Cellular life within the cardiovascular system is harsh—even under normal physiology. Unrelenting exposure to stretch, contraction, harmful metabolic byproducts, and hemodynamic forces constantly assault cells, some of which endure life spans of many decades (eg, myocytes and VSMCs). During disease, vascular cells are further exposed to a new palette of insults, many of which trigger cell death. What is becoming increasingly clear is that not only does direct loss of a cell have consequence, but how neighboring and infiltrating cells respond also affects pathology. The local milieu has multiple pro- and antiapoptotic influences and can inhibit phagocytosis of dead cells, leading to necrosis. Release of DAMPs from even modest numbers of necrotic cells can induce inflammation, perpetuating chronic inflammation. How these multiple pathways integrate in vivo is being elucidated, and their regulation may lead to future development of effective therapies.

**Disclosures**

None.

**References**


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