Apothotic Cell Death and Efferocytosis in Atherosclerosis

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Abstract—Apothotic cell death is an important feature of atherosclerotic plaques, and it seems to exert both beneficial and detrimental effects depending on the cell type and plaque stage. Because late apoptotic cells can launch proatherogenic inflammatory responses, adequate engulfment of apoptotic cells (efferocytosis) by macrophages is important to withstand atherosclerosis progression. Several efferocytosis systems, composed of different phagocytic receptors, apoptotic ligands, and bridging molecules, can be distinguished. Because phagocytes in atherosclerotic plaques are very much solicited, a fully operative efferocytosis system seems to be an absolute requisite. Indeed, recent studies demonstrate that deletion of just 1 of the efferocytosis pathways aggravates atherosclerosis. This review discusses the role of apoptosis in atherosclerosis and general mechanisms of efferocytosis, to end with indirect and direct indications of the significance of effective efferocytosis in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2012;32:887-893.)

Key Words: apoptosis ▪ atherosclerosis ▪ macrophages ▪ efferocytosis

Histological analysis of advanced atherosclerotic lesions demonstrates the presence of large necrotic and acellular regions.1 Although cell death in atheromata may result from lytic injury and oncosis (gain in cell volume, swelling of organelles, plasma membrane rupture, and subsequent loss of intracellular contents), it was proposed that most of the cells in atheromata undergo apoptosis.2 Apoptosis is a physiological, programmed, and energy-dependent cell death cascade (reviewed in3), wherein a set of cysteine aspartate-specific proteases, named caspases, gradually dismantle the dying cell. The term “apoptosis” (from the Greek word “falling”) was coined by Kerr et al in 19724 to describe a morphological distinct type of cell death. Apoptosis is accompanied by rounding up of the cell, retraction of pseudopods, reduction of cellular volume, chromatin condensation, nuclear fragmentation, little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing (but maintenance of its integrity until the final stages of the process), and engulfment by resident phagocytes (in vivo). These apoptotic features are detected in atherosclerotic plaques by light and transmission electron microscopy. Other evidence for apoptosis includes the detection of the DNA laddering produced by cleavage of genomic DNA, terminal deoxynucleotidyl transferase dUTP nick-end labeling, and detection of caspase-3 activation or cleavage of poly(ADP-ribose)polymerase-1 in atherosclerotic lesions.3 Although apoptotic cells (ACs) are studied form of cell death in atherosclerotic plaques. Autophagy contributes to cellular recovery in an adverse environment, but its functional significance in atherosclerosis remains unclear, and adequate routine markers are still lacking.8

Implications of Apoptosis in Atherosclerotic Plaques

It has become evident that apoptosis is implicated in the progression of atherosclerosis. Indeed, the level of AC death is strongly related to atherosclerotic lesion stage and plaque rupture.9 In general, adaptive intimal thickenings and fatty streaks contain very few ACs, whereas advanced lesions consist of apoptotic foci.5 Several factors implicated in atherosclerosis or present in plaques have been shown to induce apoptosis, including oxidative stress, hypoxia,
Apoptosis and efficient efferocytosis in early atherosclerosis: protective

Apoptosis and defective efferocytosis in advanced atherosclerosis: detrimental

interferon-γ, and cholesterol overload (in case of macrophages).³ Apoptosis can affect all the cell types in the atherosclerotic plaque: vascular cells, such as endothelial¹⁰ and smooth muscle cells,³ as well as inflammatory cells, such as T-lymphocytes and macrophages.³ The consequences of apoptosis within atherosclerotic plaques will depend on the cell type that is involved, the plaque stage, and apoptotic cell localization. Death of endothelial cells, in contact with the blood flow, may initiate plaque erosion, resulting in clinical events because of thrombosis in the absence of plaque rupture.¹⁰ Regarding smooth muscle cells, apoptosis could destabilize the fibrous cap and induce rupture.¹¹ Macrophages represent the majority (more than 40%) of dead cells in the atherosclerotic lesions.⁹ The studies on the modulation of macrophage apoptosis during atherosclerosis development seem controversial at first glance. Some animal studies strongly suggest that macrophage apoptosis is a negative regulator of plaque growth. Transplantation of low-density lipoprotein (LDL) receptor⁻/⁻ mice with bone marrow of apoptosis resistant bax⁻/⁻, or apoptosis signal-regulating kinase 1⁻/⁻ mice induced an increase of vascular lesion size. Also G2a⁻/⁻apolipoprotein E (ApoE)⁻/⁻ mice that have decreased macrophage apoptosis and therefore an accumulation of lesional macrophages demonstrate increased atherosclerosis.¹⁴ Selective inhibition of the antiapoptotic gene Bcl-2 in macrophages resulted in increased apoptosis and plaque necrosis in advanced lesions.¹⁵ However, other experiments suggest that macrophage apoptosis promotes necrotic core formation and atherosclerosis. C/EBP homologous protein⁻/⁻ mice have less macrophage apoptosis in response to cholesterol-induced endoplasmic reticulum stress and have reduced necrotic core and lesion development in both ApoE⁻/⁻ and LDL receptor⁻/⁻ background,¹⁶ whereas ApoE⁻/⁻ mice with heterozygosity for a protein involved in cholesterol trafficking demonstrate decreased macrophage apoptosis, lesional necrosis, and atherosclerosis, suggesting that macrophage apoptosis increases plaque progression.¹⁷ These contradictory observations might be explained by different effects of macrophage apoptosis depending on atherosclerotic plaque stage, as was illustrated by Gautier et al.¹⁸ and schematically represented in the Figure. In the model of Gautier et al, an increase of macrophage apoptosis susceptibility induced a decrease of plaque size after 5 weeks of Western diet but an increase after 15 weeks of Western diet.¹⁸ Also, Babaev et al demonstrated that increased macrophage apoptosis reduces early atherosclerosis.¹⁹ We could speculate that macrophage apoptosis in early lesions would limit plaque development through a negative regulation of inflammation. In more advanced plaques, macrophages and foam cells are specialized in and necessary for AC clearance and scavenging in a microenvironment where efferocytosis mechanisms are compromised (see below). Subsequently, their death will further alter cellular debris removal and promote plaque growth.

On top of this, other studies demonstrate a major role for apoptosis in the thrombogenicity of atherosclerotic plaques. First, apoptotic intimal cells in plaques are a source of tissue factor,²⁰ a potent inducer of the coagulation cascade. In addition, ACs externalize phosphatidylserine to their outer cell membrane, thereby enhancing tissue factor activity.²¹ In atherosclerotic lesions ACs colocalize with tissue factor, whereas shed apoptotic bodies from plaques show procoagulant activity,²² which can stimulate thrombosis locally as well as systemically. Finally, unstable lesions contain more tissue factor than stable lesions, mostly in macrophage-rich regions close to the necrotic core,²³ and are associated with increased plaque thrombogenicity.²⁴ Furthermore, atherosclerotic plaques from diabetic patients, which are known to be more unstable than those of nondiabetic patients, are rich in apoptotic debris.²⁵

**Clearance of ACs: Mechanisms of Efferocytosis**

Apoptosis is rarely observed in normal histological sections because prompt clearance mechanisms narrow the observa-
tional window. Indeed, not only “professional” phagocytes, such as macrophages and dendritic cells (DCs), but also “amateur” neighboring scavengers (sometimes of the same lineage), including endothelial cells, smooth muscle cells, and fibroblasts, are capable of taking up ACs. “Efferocytosis” (Greek for “carrying the corpse to the grave”) was introduced as a term to specifically refer to the engulfment or phagocytosis of ACs.\(^6\) Hence, the distinction is made with phagocytosis of, for example, pathogens, in which the mechanisms of uptake, intracellular signaling, and subsequent immune responses are very different. Several phases can be distinguished in efferocytosis: attraction of phagocytes, recognition, engulfment, and postengulfment responses.

When cells undergo apoptosis they release soluble attraction or “find me” signals to recruit phagocytes. One such signal is the lipid lysophosphatidylcholine, which is formed by the breakdown of the membrane glycerolipid phosphatidylcholine by caspase-3-activated phospholipase A2. The G-protein coupled receptor G2a is proposed as its in vivo receptor. Besides lysophosphatidylcholine, phagocytes can sense ACs by a gradient of the nucleotides ATP and UTP (P2Y2 purinergic receptor), sphingosine-1 phosphate and its receptor, and CX3CL1/fractalkine (CX3C receptor). A more complete list of “come and get me” factors can be found in the recent review by Peter et al.\(^27\) Furthermore, microblebs released from ACs are implicated in long distance communication and attraction of phagocytes.\(^28\) Adding to this, it was recently shown that microRNA-126 delivered by apoptotic bodies can transmit paracrine signals, resulting in attraction of progenitor cells and limited atherosclerosis.\(^29\) The leaky cell membrane of necrotic or late ACs liberates additional proteins, so-called alarmins, which propagate a danger signal and alert the immune system.\(^6,27\) Examples of alarmins that are exclusively released by primary necrotic cells include the nDNA binding protein high-mobility group box 1 protein and heat shock proteins, which can bind several Toll-like receptors.\(^6,27\) Interleukin-1,\(^30\) uric acid, SIN3A-associated protein 130 (SAPI30, which binds to the macrophage-inducible C-type lectin receptor MINCLE/CLEC4E),\(^6,27\) and a yet unidentified alarmin that is recognized by another C-type lectin receptor (CLEC9A) on CD8α DCs are potential attraction mechanisms used by primary as well as secondary necrotic cells.\(^6,27\) Interestingly, several of these alarmins, such as high-mobility group box 1 protein,\(^31\) interleukin-1,\(^32\) and heat shock proteins,\(^32\) have been implicated in atherosclerosis. Finally, changes in the membrane composition of ACs result in an altered capacitance.\(^33\) Consequently, ACs may initiate electric signals that attract phagocytes, as has been described for endothelial cells.\(^34\) As recently reviewed,\(^35\) in atherosclerosis an overload of these “eat me” signals can be found.

In addition to “find me” signals, ACs will express “eat me” signals, i.e., molecules that are upregulated or translocated to the surface to facilitate interactions with phagocytes. In fact, efferocytosis will depend on the quantity and quality of positive (“eat me”) and negative (“don’t eat me”) signals. Healthy cells protect themselves from engulfment by expressing CD31 and CD47,\(^6\) which repulse phagocytes. Meanwhile, the plasma membrane architecture of ACs is very different from that of viable cells. One of the earliest changes in is the translocation of phosphatidylserine (PS) to the outer face of the cell. PS on ACs is mostly present in patches and sometimes in oxidized forms.\(^30\) Several phagocytic receptors are supposed to bind PS, including brain specific angiogenesis inhibitor 1 and T-cell immunoglobulin mucin receptor.\(^6\) However, recently it was shown that PS exposure by itself does not suffice for adequate efferocytosis.\(^37\) Several bridging molecules facilitate the interaction of PS on the AC with receptors on phagocytes. Among them are the plasma protein β2 glycoprotein, which binds the β2 glycoprotein receptor; growth arrest–specific growth factor-6 and protein S, which form the bridge between PS and tyro3/axl/mer receptor tyrosine kinase family (MER, Tyro3, Axl); and milk fat globule epidermal growth factor (MFGE8), which connects PS and the integrins α,β₃ and α,β₅.\(^6,26\) The oxidized lipid-proteins on apoptotic plasma membranes will bind to scavenger receptors (SR-A, LOX-1, CD68, CD14, CD36) on phagocytes. Several molecules (eg, complement factors C1q, iC3b, pentraxins, thrombospandin-1) were shown to opsonize ACs and facilitate the interaction with pattern recognition receptors on phagocytes.\(^5\)

In the end, the “phagocytic synapse” tightly connects the phagocyte with the dying cell (zipper like interaction). After ingestion by the phagocyte, the internalized AC will be processed and degraded through a series of fusions with endosomes and lysosomes.\(^6\) Subsequent presentation of antigens by macrophages or DCs enables the induction of adaptive immune responses. Recently, it was demonstrated that the mitochondrial membrane protein uncoupling protein 2 (Ucp2), which acts to lower the mitochondrial membrane potential, is a critical efferocytosis regulator in phagocytes.\(^38\) Indeed, ingestion of ACs alters phagocyte mitochondrial membrane potential. Absence of Ucp2 compromises engulfment of ACs in vitro and in vivo, whereas overexpression of Ucp2 enhances efferocytosis.\(^38\) These results are consistent with the marked accumulation of ACs, the increase of necrotic core, and the acceleration of atherosclerosis in Ucp2-deficient mice\(^39\) and further emphasize the importance of adequate efferocytosis in atherosclerosis.

The effect of cell death on the immune system is discussed by Zheng et al.\(^40\) In many occasions, ACs were shown to produce anti-inflammatory factors, as well as directly induce tolerogenic responses in antigen presenting cells,\(^41\) through the release of anti-inflammatory cytokines such as interleukin-10 and transforming growth factor-β.\(^7\) Meanwhile, primary and secondary necrotic cells are associated with inflammation. Nevertheless, there still remains controversy regarding these assumptions.\(^5–7\) Generally, the nature of the immune response to apoptosis and its subsequent impact on atherogenesis will depend on the intrinsic antigenicity of the cells, the history of activation or stress before cell death, the nature of the cell death inducer, the cell death pathway that is engaged, and the availability of phagocytes and immune cells that can respond.\(^5\) Indeed, though many tolerogenic responses are attributed to ACs, some ACs can exert proinflammatory effects,\(^5\) as is described for ACs with oxidative epitopes.\(^32\) Another illustration of diverse immune responses to ACs is the observation that free cholesterol-
induced apoptosis of macrophages results in a proinflammatory response, whereas oxidized LDL (oxLDL)–induced or UV-induced apoptosis results in a more tolerogenic immune response.\textsuperscript{43} Finally, as mentioned above, the impact of apoptosis is beneficial in early stages of atherosclerosis, whereas in advanced lesions, it contributes to plaque instability and thrombogenicity.\textsuperscript{18} It is assumed that less efficient scavenging of ACs and subsequent secondary necrosis play a role in unstable atherosclerotic lesions.

**Impaired Efferocytosis in Atherosclerosis**

Macrophages containing abundant apoptotic material are detected in plaques, supporting that efferocytosis occurs in atherosclerosis. Yet the occurrence of apoptotic debris extracellularly and the very high numbers of free ACs encountered in atherosclerotic lesions\textsuperscript{44} suggest either increased AC death or insufficient mechanisms for the removal of ACs in atherosclerosis. It should be acknowledged that studies on efferocytosis in atherosclerosis are based on microscopic analysis of AC and phagocytes colocalization in histological sections. This might be hampered by difficult discrimination between AC adherence compared with uptake by efferocytes. Moreover, conclusions are merely based on observations and associations at a certain time point because functional data on in vivo kinetics of AC clearance in plaques are difficult to obtain.

Increased cell death may result from the omnipresence of apoptotic triggers such as oxLDL and interferon-\(\gamma\), especially in advanced atherosclerotic lesions, but sufficient numbers of phagocytes seem to be present and colocalize with ACs in plaques. Alternatively, several in vitro studies suggest a major role for impaired efferocytosis in atherosclerosis. For instance, competition between ACs and oxidized lipids for clearance by phagocytes was repeatedly observed.\textsuperscript{44,45} Moreover, infusion of lysophosphatidylcholine reduced AC uptake in gld mice, which demonstrate higher AC levels because of genetically impaired Fas-ligand.\textsuperscript{46}

Because ACs and oxidized lipids share antigenic properties, autoantibodies directed against oxLDL also fix on ACs and are able to inhibit their engulfment.\textsuperscript{47} However, a more recent in vivo study demonstrated that natural IgM against oxLDL might not inhibit but facilitate the uptake of ACs by macrophages.\textsuperscript{47} Moreover, Ogden et al showed that IgM is required for optimal complement dependent efferocytosis of ACs.\textsuperscript{48} Besides competitive inhibition with oxLDL, oxidative stress\textsuperscript{44} and transformation into foam cells\textsuperscript{49} also impair the capacity to engulf apoptotic debris. Interestingly, the modulation of cholesterol transport in macrophages affects the expression of molecules implicated in efferocytosis such as MFGE8.\textsuperscript{49} Moreover, using immunohistochemistry, we have demonstrated a decrease of MFGE8 protein expression in macrophages around the necrotic core.\textsuperscript{50} These data suggest that changes in macrophage phenotype during atherogenesis and foam cell formation might substantially alter efferocytosis. Furthermore, it was shown that increased fatty acids in macrophage membrane lipid composition can cause impaired efferocytosis. This was illustrated in obese ob/ob LDL receptor\textsuperscript{\textsuperscript{−/−}} mice in which a fish oil–rich diet reversed the effects.\textsuperscript{51} Finally, inflammation is also likely to have profound effects on efferocyte function. For instance, mertyrosine kinase (MERTK) is cleaved under inflammatory conditions, and soluble MERTK can compete for the ligands of membrane bound MERTK and therefore impede efferocytosis.\textsuperscript{52}

Apart from macrophages, increased numbers of immature DCs are present in atherosclerotic lesions\textsuperscript{53} and might also be implicated in efferocytosis in atherosclerosis. As recently discussed,\textsuperscript{54} different efferocytes might have different effects on plaque evolution. DCs are less well equipped than macrophages to digest internalized proteins but are more specialized in the overall interpretation of the microenvironmental signals, the induction of adequate immune responses after AC uptake, and the migration to lymphoid organs. Interestingly, ACs contained in DC phagosomes can be found in afferent lymphatics.\textsuperscript{55} Immature DCs are specialized in uptake of substances but lose their phagocytic capacity after maturation as soon as their antigen presenting functions are induced.\textsuperscript{56} As for macrophages, they are considered less migratory but could transform into DCs to transport cell death material to lymphoid organs.\textsuperscript{57} Although in vitro studies have shown that AC uptake can induce tolerogenic responses in the phagocyte,\textsuperscript{51} at the moment, studies on efferocytosis by different phagocytes in atherosclerotic plaques are lacking, and it is unknown whether they undergo a phenotypic transformation, alter their function, and emigrate from the plaque after AC uptake.

It is widely assumed that delayed clearance of ACs leads to secondary necrosis, membrane leakage of inflammatory cell contents, and triggering of autoimmunity. Although mice lacking the phagocytic receptor CD14 challenge this hypothesis by showing an accumulation of apoptotic bodies without autoimmune pathogenesis or autoantibodies.\textsuperscript{58} Other mouse models of decreased apoptosis engulfment, such as mice lacking the receptor MER\textsuperscript{59} and the bridging molecules MFGE8\textsuperscript{50,61} and Clq,\textsuperscript{62} do demonstrate an association between increased nonphagocytosed apoptotic bodies and autoimmune pathology with increasing age. Therefore, it was suggested that besides their role in efferocytosis, signaling through the MER receptor or integrins via MFGE8 is required for direct immune suppressive effects following AC recognition.\textsuperscript{63} Interestingly, MER attenuated the inflammatory response to free cholesterol loading of macrophages in vitro\textsuperscript{43} and ligated tyro3/axl/mer receptors negatively regulate inflammatory pathways through the induction of suppressors of cytokine signaling proteins.\textsuperscript{64} Also, MFGE8 directly influences regulatory immune responses.\textsuperscript{50,65} Finally, other evidence that clearance of ACs is important for the maintenance of immune tolerance is seen for the receptor peroxisome proliferator–activated receptor-\(\delta\), which is induced when macrophages engulf ACs. Deletion of peroxisome proliferator–activated receptor-\(\delta\) results in decreased expression of opsonins, such as the complement factor Clq, MFGE8, and MERTK, impaired efferocytosis and autoimmune responses in mice.\textsuperscript{66} Similarly, liver X receptor (LXR) is induced by AC uptake and enhances MERTK expression. LXR deficiency results in impaired efferocytosis and increased inflammation.\textsuperscript{67}

Moreover, it is probable that in atherosclerotic lesions, which are already a proinflammatory milieu in which engul-
ment mechanisms are very much solicited, impairment of efferocytosis pathways will have more pronounced effects. Indeed, we believe that atherosclerotic lesions represent a very particular, pathological microenvironment in which very high apoptosis rates demand a fully functional and available efferocytosis system. Therefore, blockage of just 1 efferocytosis pathway in atherosclerosis is likely to have much more extensive effects. Indeed, this was illustrated by recent studies analyzing deficiency of either MFGE8, MERTK, or C1q efferocytosis pathways in models of atherosclerosis.46,50,68–70 In these studies, invalidation of only 1 pathway had strong effects on atherosclerosis with enhanced accumulation of ACs, even in early lesions, an increase of necrotic core, and in some cases an increase in plaque size (as illustrated in the Figure). We showed that the absence of the bridging molecule MFGE8 in a mouse model of atherosclerosis leads to accelerated atherosclerosis with enlarged necrotic cores, because of an accumulation of AC debris. In addition, MFGE8 deficiency coincided with a switch of the immune response toward a Th1 proinflammatory phenotype.50 Furthermore, studies by Tabas’s group68 and ours69 showed that defect in the phagocytic receptor MER leads to increased accumulation of ACs, resulting in enhanced plaque necrosis, a proinflammatory phenotype, and aggravated atherosclerosis. Finally, the complement factor C1q was also shown to be an essential bridging molecule for efficient efferocytosis and limited atherosclerosis.70 Other experimental studies indicating the importance of defective efferocytosis in atherosclerosis include mouse models that might undergo increased apoptosis susceptibility in addition to deficient AC engulfment. Even so, G2a-deficient mice with decreased macrophage apoptosis as well as efferocytosis demonstrate increased atherosclerosis.14 Furthermore, inactivated Fas-ligand in ApoE−/− mice (gld ApoE−/− mice) resulted in more ACs, less efferocytosis, and enhanced atherosclerosis.96 Besides, deficiency in ApoE itself modulates clearance in vitro and in vivo independently of its role in lipoprotein metabolism,73 potentially adding to the increased susceptibility of ApoE−/− mice to develop atherosclerosis. Finally, transglutaminase-2–deficient macrophages demonstrate deficient AC ingestion in vitro, whereas transglutaminase-2–deficient bone marrow resulted in increased atherosclerosis in mice.72

Of note, the above-described observations on impaired efferocytosis in atherosclerosis can be of interest for other major autoimmune diseases, such as lupus erythematosus, which is also characterized by increased cell death and defective efferocytosis. As in atherosclerosis, inadequate engulfment and digestion of dead cells may result in secondary necrosis, improper presentation of antigens, and subsequent chronic activation of the immune system. Interestingly, patients with lupus erythematosus are at increased risk to develop cardiovascular events.73

**Concluding Remarks**

Along with cell proliferation and apoptosis equilibriums, clearance of dying/dead cells is vastly important for the development and homeostasis of organisms. Endogenous components secreted from or expressed on dead cells can induce innate and adaptive immune responses. It seems that the complex, debris-rich environment of the advanced atherosclerotic lesion challenges the many engulfment mechanisms. Indeed, deletion of efferocytosis pathways leads to aggravated inflammation and increased atherosclerosis.

The current knowledge on the many molecular pathways of efferocytosis, the different efferocyte subsets (macrophages and DCs), and their interaction with the immune system require further exploration to better understand the determinants of plaque progression, as well as plaque instability. In the end, this will allow us to identify new therapeutic targets to treat atherosclerosis. As discussed above, ACs can induce peroxisome proliferator–activated receptorα and LXR receptors, which result in upregulation of opsonins and efferocytosis-related pathways, such as C1q or MERTK receptor. Consequently, administration of ligands to peroxisome proliferator–activated receptorα or LXR might become a therapeutic strategy to promote efferocytosis. Interestingly, administration of a synthetic agonist for LXR did ameliorate lupus autoimmunity in a mouse model,67 suggesting that at least part of the beneficial effect of LXR agonists in cardiovascular disease might be related to their efferocytosis-dependent immunomodulatory effect.

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**Disclosures**

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