Macrophage Apoptosis in Advanced Atherosclerosis

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Plaque necrosis in advanced atheromata, which triggers acute atherothrombotic vascular events, is caused by the apoptosis of lesional macrophages coupled with defective phagocytic clearance of the dead cells. The central enabling event in macrophage apoptosis relevant to advanced atherosclerosis is the unfolded protein response (UPR), an endoplasmic reticulum (ER) stress pathway. The UPR effector CHOP (GADD153) amplifies release of ER Ca^{2+} stores, which activates a central integrator of apoptosis signaling, calcium/calmodulin-dependent protein kinase II (CaMKII). CaMKII, in turn, leads to activation of pro-apoptotic STAT1, induction of the death receptor Fas, and stimulation of the mitochondria-cytochrome c pathway of apoptosis. While these pathways are necessary for apoptosis, apoptosis occurs only when the cells are also exposed to one or more additional "hits." These hits amplify pro-apoptotic pathways and/or suppress compensatory cell-survival pathways. A second hit relevant to atherosclerosis is activation of pattern recognition receptors (PRRs), such as scavenger and toll-like receptors. *In vivo* relevance is suggested by the fact that advanced human lesions express markers of UPR activation that correlate closely with the degree of plaque vulnerability and macrophage apoptosis. Moreover, studies with genetically altered mice have shown that ER stress and PRR activation are causative for advanced lesional macrophage apoptosis and plaque necrosis. In summary, a key cellular event in the conversion of benign to vulnerable atherosclerotic plaques is ER stress-induced macrophage apoptosis. Further understanding of the mechanisms and consequences of this event may lead to novel therapies directed at preventing the clinical progression of atheromata.

Key words: **atherosclerosis; macrophage; apoptosis; plaque necrosis; unfolded protein response; calcium/calmodulin-dependent protein kinase II**

Introduction

Almost all subjects over the age of 20 in industrialized societies have atherosclerotic lesions in their coronary arteries and other susceptible sites.[1] However, only a very small minority of these lesions will progress to a stage where they can precipitate acute vascular events like myocardial infarction, sudden cardiac death, and stroke.[2] Therefore, preventing the progression of so-called benign lesions to "vulnerable" plaques may afford a focused approach to prevent the *consequences* of atherosclerosis as opposed to atherogenesis itself. Interestingly, the lesions that tend to cause acute clinical disease are not necessarily the largest lesions but rather those that have certain features that can trigger acute thrombotic vascular occlusion.[3] Among these features are large collections of dead macrophages known as necrotic cores.[4]

The infiltration of blood-borne monocytes into nascent atherosclerotic lesions is one of the earliest responses to the subendothelial matrix retention of apolipoprotein B lipoproteins, which is the universal initiating event of

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atherosclerosis.[1,5,6] The monocytes differentiate into macrophages in the subendothelium and begin to ingest the retained lipoproteins. Eventually, the macrophages become so full of lipoprotein-derived cholesterol, which they store as cytoplasmic cholesteryl ester droplets, that they take on a "foamy" appearance and thus are known as "foam cells."[1,5,6] Genetic studies in mice have provided evidence that early lesional foam cells promote lesion progression through a number of processes that are still being investigated.[7]

As lesions progress, signs of macrophage apoptosis begin to appear.[8] In normal physiology, many cells throughout the body undergo apoptosis each day. However, rapid phagocytic clearance of the corpses ("efferocytosis") prevents subsequent cell leakage, known as postapoptotic necrosis, and actually activates anti-inflammatory cell-signaling pathways.[9] Indeed, there is evidence that macrophage apoptosis occurs in early lesions, but efficient efferocytosis renders this process not only harmless but possibly beneficial.[10] In advanced lesions, however, macrophage apoptosis is associated with secondary necrosis, suggesting defective efferocytosis.[10,11] For example, Schrijvers and Martinet[12] showed that there are large numbers of nonengulfed apoptotic cells in advanced human atheromata, compared with a control tissue, human tonsils. When these data are considered together with the overall concept of postapoptotic necrosis and the observation that advanced lesional necrotic cores are filled with macrophage debris,[13] macrophage apoptosis coupled with defective efferocytosis provides a plausible model for the genesis of plaque necrosis.

Macrophage Apoptosis Signaling Pathways Relevant to Advanced Atherosclerosis

Whereas the mechanism of defective efferocytosis in advanced lesions is not known, studies over the last several years have revealed important principles related to mechanisms of advanced lesional macrophage apoptosis. Work in our laboratory focused first on an observation that pathologists have made for decades, namely, that macrophages in advanced lesions accumulate increasing amounts of unesterified, or "free," cholesterol (FC), as opposed to the typical storage form in early lesional foam cells, cholesteryl esters.[14] Although the mechanism of FC accumulation *in vivo* is not known, we and others have set up experimental cell culture models of FC-loaded macrophages by using inhibitors and null mutations of the cholesterol esterifying enzyme, acyl-CoA:cholesterol acyl transferase (ACAT).[15–17] FC-loaded macrophages were found to undergo apoptosis through classical Fas death receptor and mitochondrial pathways of apoptosis.[16,17]

Ensuing mechanistic studies in our laboratory revealed that the key event in FC-induced apoptosis was transfer of excess FC to the endoplasmic reticulum (ER) membrane.[18] The ER membrane evolved as a relatively fluid, cholesterol-poor bilayer, and excess cholesterol causes the membrane to become more "stiff."[19] When this happens, the cells respond by activating a highly conserved, integrated signal transduction pathway called the unfolded protein response (UPR).[18] The UPR is activated in cells when the delicate homeostasis of ER function is disturbed,[20,21] as is clearly the case with FC enrichment of the ER membrane.[19] The UPR leads to the induction of many proteins, such as protein chaperones, that serve to correct the disequilibrium in the ER.[20,21] However, when such correction fails due to severe or prolonged ER stress, a consequence of prolonged UPR activation is apoptosis.[22,23] The mechanisms of ER stress-induced apoptosis have been the subject of many articles and reviews in areas spanning the gamut of pathology, including apoptosis of neurons, pancreatic beta cells, and cancer cells.[23] Nonetheless, an integrated paradigm for how prolonged UPR activation triggers specific apoptosis pathways has not clearly emerged.

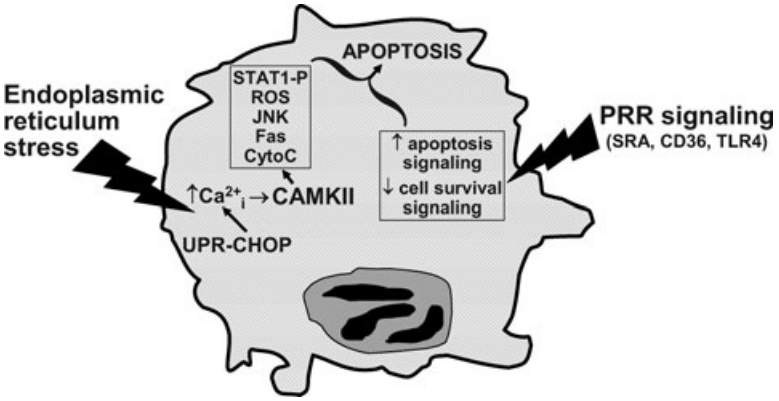

Figure 1. The ER stress/PRR model of macrophage apoptosis. Apoptosis occurs only when macrophages are subjected to both ER stress and a second hit, which here is depicted as pattern recognition receptor (PRR) signaling. The ER stress hit, through direct effects on the ER and through CHOP induction, results in release of ER calcium stores into the cytoplasm ($Ca^{2+}i$). Cytoplasmic calcium activates CaMKII, which induces a number of pro-apoptotic processes, including STAT1 phosphorylation, reactive oxygen species (ROS) generation, Jun N-terminal kinase (JNK) activation, Fas induction, and cytochrome c (CytoC) release from the mitochondria. The PRR hit amplifies some of the apoptotic pathways (such as activation of STAT1) and suppresses compensatory cell survival pathways (such as induction of interferon-β). See text and references therein for details.

Over the last several years, we have put together a scheme of apoptosis in ER-stressed macrophages that appears to be highly relevant to advanced atheromata and may be applicable to other scenarios of UPR-induced apoptosis. It is important to note that our studies have taken us beyond the FC model into one that applies more generally to ER stressors, including those that are known to be present in advanced atherosclerotic lesions (below). The scheme can be summarized as follows (Fig. 1). The key initiating event in the apoptosis cascade in ER-stressed macrophages is release of calcium from ER stores into the cytosol.[24,25] Many ER stressors, including FC loading, are known to precipitate this event through inhibition of the ER calcium reuptake pump, sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), and/or by activation of ER calcium channels, notably inositol-3-phosphate receptors (IP3R).[26] In addition, very recent work in the laboratory has revealed a novel pathway whereby the UPR itself, through its downstream effector C/EBP-homologous protein (CHOP) growth arrest and DNA-damage-inducible gene 153 (GADD153), amplifies ER calcium release (Li and colleagues, manuscript in preparation). We found that cytosolic calcium is necessary for ER stress-induced apoptosis in macrophages.[24,25] Moreover, this requirement for calcium can be explained mostly, if not entirely, through activation of calcium/calmodulin-dependent protein kinase II (CaMKII).[25] Ongoing work in the laboratory is elucidating how CaMKII signaling triggers apoptosis. Our data thus far suggest that CaMKII is a central integrator of pro-apoptotic processes, including induction of Fas, activation of the signal transducer and activator of transcription-1 (STAT1), stimulation of cytochrome c release from the mitochondria, and generation of reactive oxygen species (ROS)[25,27] (Li and colleagues, submitted manuscript).

A fundamental principle that has arisen from our studies on ER stress-induced apoptosis is the concept of the "two-hit model."[24,28] Despite the array of pro-apoptotic processes triggered by ER stress (above), this alone does not cause apoptosis in our model.[28] Surely, one

can induce apoptosis *in vitro* by subjecting the cells to very high levels of ER stressors, but we do not consider this situation relevant to what happens *in vivo*, even under pathological conditions. On the other hand, when macrophages are subjected to sublethal ER stress, apoptosis can be induced by "second hits" that are also nonapoptotic by themselves. For example, we have focused on activators of pattern recognition receptor (PRR) signaling in macrophages. PRRs are cell-surface receptors that respond to a wide array of pathogen-associated molecules, such as bacterial lipids and viral nucleic acids.[29] PRR signaling leads to induction of antimicrobial and inflammatory responses that are essential to the process of innate immunity.[29] However, in the case of ER-stressed macrophages, activation of a number of PRR receptors, including the type A scavenger receptor, CD36, and toll-like receptor 4 (TLR4), trigger apoptosis[24,28] (Seimon and coworkers, unpublished data). The mechanisms are still being investigated, but in general PRR activation amplifies the aforementioned pro-apoptotic pathways and/or suppresses compensatory cell-survival pathways.[24]

Evidence for the ER Stress/PRR Model of Macrophage Apoptosis in Advanced Atheromata

There are increasing data suggesting that the ER stress/PRR model of macrophage apoptosis is relevant to macrophage death and subsequent plaque necrosis in advanced atheromata. First, all of the "players" in the pathways—UPR activators, PRRs and their ligands, and pro-apoptotic signaling molecules—are present in macrophage-rich regions of advanced atheromata.[30–38] Second, there is now clear evidence that advanced lesional macrophages actually undergo ER stress *in vivo*.[18,39–41] For example, a recent study by Myoishi and colleagues[41] has shown that advanced human coronary artery plaques express UPR markers in a manner that correlates closely with lesional macrophage apoptosis and plaque vulnerability. Third, a number of the pathways or molecules in the model have been genetically altered in mice, and careful analysis of advanced lesions for macrophage apoptosis and plaque necrosis has yielded data that are consistent with causation. For example, a genetic manipulation that blocks cholesterol trafficking to the ER, and thus FC-induced UPR activation and apoptosis, suppresses macrophage apoptosis and plaque necrosis in advanced lesions of $Apoe^{-/-}$ mice.[42] Similar results were obtained with $Apoe^{-/-}$ or $Ldlr_{-/-}$ mice lacking signal transducer and activator of transcription 1 (STAT1),[25] CHOP[43] or scavenger receptors.[44] Moreover, cell culture studies revealed that macrophages with defective insulin signaling, which occurs in insulin-resistant states, have a heightened UPR response and suppressed compensatory cell-survival signaling and are more susceptible to ER stress/PRR-induced apoptosis.[45] *In vivo*, advanced lesions of $Ldlr^{-/-}$ mice reconstituted with macrophages having defective insulin signaling show enhanced macrophage apoptosis and plaque necrosis.[45] This study may be highly relevant in view of the ongoing epidemic of insulin resistance–associated coronary artery disease—disease associated with particularly large areas of plaque necrosis in the coronary and carotid arteries.[46]

Summary

We have elucidated a pathway of macrophage apoptosis that involves known molecules and processes in advanced atheromata and that is consistent with a growing gallery of correlative human evidence and causative animal data for advanced lesional macrophage apoptosis and plaque necrosis. Given that most patients enter the medical system for primary or secondary prevention therapy *after* atherosclerosis is already well developed, we feel our studies have the potential for a new type of therapeutic strategy, namely, one aimed at preventing the progression of plaques to the vulnerable stage

rather than preventing atherogenesis *per se*. Of course, such therapy would only make sense when combined with proven risk-reduction approaches, particularly the lowering of plasma non-HDL cholesterol.[1] Moreover, one would imagine that such therapy would be highly complementary to ongoing strategies to promote plaque regression through enhancement of lesional cellular cholesterol efflux and reverse cholesterol transport.[47] The aging of the population and the aforementioned epidemic of obesity, insulin resistance, and type 2 diabetes demand consideration of multiple approaches to combat what is soon predicted to be the leading killer worldwide.

Conflicts of Interest

The authors declare no conflicts of interest.

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