Role of Caspases in Death and Survival of the Plaque Macrophage

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Abstract—This review considers the role of macrophage cell death in formation of the necrotic core and in plaque progression, and lists many of the possible mediators of macrophage cell death. Among these, perhaps the most cited toxic agent is oxidized low-density lipoprotein (oxLDL). Whereas oxLDL can kill macrophage, and whereas the form of death is morphologically apoptotic, caspase inhibitors appear to be ineffective in preventing death. This finding is consistent with recent literature showing how the canonical caspase pathways are used for physiological cellular functions other than cell death. Plaque macrophages appear to be among the cells with this nonapoptotic signaling function for activated caspases. In many of the other cell types, caspase activation appears to play a critical role in cell differentiation. We discuss possible functions of plaque macrophage using the nondeath caspase pathway. Recent literature shows that physiological and developmental functions of many cell types require active caspases without progressing to cell death. We discuss the role of macrophage cell death in plaque progression, possible mediators of macrophage cell death, and the possible functions of plaque macrophage using the nondeath caspase pathway. (Arterioscler Thromb Vasc Biol. 2005;25:895-903.)

Key Words: apoptosis ■ caspase ■ death receptors ■ monocyte/macrophage ■ plaque rupture

Rupture of the fibrous cap would be of little consequence if it were not for exposure of the underlying necrotic core.1,2 This is not a new idea. As early as the 1850s, Virchow compared the atherosclerotic plaque to a sebaceous cyst.3 The fatty core of the plaque, to the best of our knowledge, results from death of the foam cells that formed the original lesions and from the accumulation of lipoproteins from the blood.4,5 The foam cells include macrophages and some smooth muscle cells. Macrophage death has been implicated in other aspects of lesion progression as well, including activation of tissue factor, release of apoptotic vesicles, formation of bone, synthesis of transforming growth factor (TGF)-β, synthesis of IL-10, and release of proteases.6–8 Finally, recent studies of murine lesions have shown that death of macrophages located at the margins of advanced plaques may be involved in murine models of plaque rupture9,10 (Figure 1). Although the role of macrophage death in rupture of human plaques is not known, apoptosis of macrophages in the rupture-prone shoulder regions of plaques is well known.

Obviously, it follows that the mechanisms of macrophage death have become a focus of research in atherosclerosis.11–13 Most of these studies refer to the death process as “apoptosis,” implying a specific mechanism. Evidence for “apoptosis” includes the presence of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive reaction. The TUNEL reaction is interpreted as evidence for activation of the canonical caspase-driven pathway, because DNA fragmentation is typically caused by caspase-dependent cleavage of the DNase inhibitor (DFF45/ICAD).14

In support of the theory that apoptosis is important in atherosclerosis, studies in the late 1990s identified cleaved caspases within human atherosclerotic lesions.15 Other articles presented evidence correlating the presence of the proapoptotic death receptor, Fas (CD95), and its cognate ligand, FasL (CD178), within the necrotic core.16,17 Thus, the term “apoptosis” has generally been taken to imply caspase-mediated death, possibly initiated by Fas.

If plaque macrophage death is caspase-dependent, then we would have several specific targets for therapeutic intervention in plaque progression. This review discusses the critical features of caspase-mediated death and the evidence that this pathway, or other death pathways, is critical to death of plaque macrophages. Finally, we address the recent evidence that caspases have functions in addition to their role in death (Table).

Cell Death in Caenorhabditis elegans

The current concept of caspase-mediated apoptosis emerged from a genetic analysis of programmed cell death during development of the nematode, Caenorhabditis elegans. Development of the adult nematode is dependent on the programmed cell death of exactly 131 cells. Analysis of genes determining death of these cells has identified 4 genes that...
determine this pattern of cell death in the *C. elegans* egg sack*:
CED-3, CED-4, CED-9, and EGL-1* (Figure 2). The last gene in the pathway is CED-3. CED-3 is a cysteine protease that cleaves at an aspartate residue. Caspas, including CED-3, are synthesized as zymogens capable of self-activation when oligomerized. CED-3 activation is dependent on genes coding for three proteins: CED-4, CED-9, and EGL-1. CED-4 serves to oligomerize and activate CED-3 via autocatalytic cleavage. CED-9 functions as a repressor of cell death by binding to CED-4 to prevent CED-4–mediated oligomerization of CED-3. A fourth gene, egl-1, codes for EGL-1, a homolog and an antagonist of CED-9. By binding to CED-9, EGL-1 releases CED-9–mediated repression on the cell death program, thereby releasing CED-4 to oligomerize with CED-3 to trigger self-destruction.18

**Mammalian Homologs of the C. elegans Death Genes**

There are at least 14 homologs of CED-3 in the mammalian caspase family. These cysteine proteases can be segregated into 3 classes: (1) “initiator” caspases that initiate the cell death program by receiving some pro-death signal from a receptor for a pro-death cytokine; (2) “executioner” caspases that execute death by proteolysis of proteins critical for life; and (3) caspases involved in processing of cytokines independently of causing death. Initiator caspases are typically low in abundance and are characterized by a recruiting mechanism of activation.

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*This manuscript follows the geneticists’ convention of italicizing gene names. For nematodes, genes are lowercase and proteins are uppercase as in *ced-3* and CED-3.
domain (CARD) needed for homo-oligomerization. Initiator caspases proteolytically activate the more abundant executioner caspases.\textsuperscript{19,20} Other caspases may operate outside of death. The best known member of this third class is caspase-1, also known as IL-1β-converting enzyme. IL-1β-converting enzyme proteolytically activates the pro-form of IL-1β into biologically active IL-1β.\textsuperscript{21} As is discussed, there is a body of literature that shows that active caspases can have nondeath functions.

The next component of the apoptotic pathway in \textit{C elegans} is CED-4. Only one mammalian homolog of CED-4 (Apaf-1, apoptotic protease-activating factor 1) has been identified.\textsuperscript{19} Apaf-1 is critical for death initiated by mitochondrial damage, in which it forms a complex with cytochrome-c to activate caspase-9.

More than 20 mammalian homologs of CED-9 and EGL-1 have been identified. The family is called the BCL-2 family after the first mammalian member to be identified as an oncogene in lymphoma.\textsuperscript{22,23} BCL-2 family members possess up to 4 BCL-2 homology domains (BH). The functional roles of BCL-2 proteins depend on BH3 and BH4 domains in destabilizing or stabilizing mitochondrial membrane potential.\textsuperscript{24} Proteins with all 4 BH domains, like BCL-2, stabilize mitochondrial membranes. Mechanistic studies of BCL-2 function led to the discovery that release of mammalian cytochrome-c from damaged mitochondrial membrane is required to amplify the caspase cascade by aggregation of Apaf-1 with caspase-9 to form the apoptosome.\textsuperscript{25} Mitochondria also contain another proapoptotic factor, AIF (apoptosis-inducing factor), which is released from damaged mitochondria.\textsuperscript{26} The \textit{C elegans} homolog of AIF, WAH-1, has also been shown to associate and cooperate with mitochondrial endonuclease CPS-6/Endonuclease-G to promote DNA degradation and apoptosis in a CED-3 (caspase)-dependent manner.\textsuperscript{27} In mammalian cells, AIF initiates a caspase-independent death pathway.\textsuperscript{26} Thus, cell death may be independent of active caspases even if, as suggested by studies of cholesterol loading, death is mediated by mitochondrial injury (discussed later).

### Three Types of Caspase-Dependent Death in Mammalian Systems

The paradigms usually described for apoptosis are called type I and type II.\textsuperscript{28} The paradigms are based on the classification of the mammalian caspases into initiator caspases and executioner caspases as discussed.

The classic example of type I death is death induced by Fas (CD95; Figure 3). Trimerized Fas, induced by binding of Fas ligand (CD178), forms a death-inducing signaling complex including ligand-, receptor-, and a caspase-activating complex consisting of an adaptor protein, Fas-associated death domain protein (FADD), procaspase-8, and a proteolytically inactive homolog of pro–caspase-8, c-FLIP.\textsuperscript{29} The zymogen, procaspase-8, is recruited to the death-inducing signaling complex via interaction with FADD and c-FLIP. It is then activated by hetero-oligomerization and proteolytic autoprocessing. This is followed by release of the activated form of caspase-8 to the cytoplasm. The activated initiator caspase-8 proteolytically activates the downstream executioner caspases, including, but not limited to, caspase-3, caspase-6, and caspase-7. Autocatalysis of the executioner caspases initiates a catastrophic proteolytic cascade (Figure 3). Although originally described in the immune system, type I death is now recognized to play an important physiological role in cell death in the liver, heart, and hematopoietic system.\textsuperscript{30} As is discussed, type I death has also been implicated in plaque rupture.

The type II paradigm also derives from studies of Fas. In this model, production of caspase-8 is limited by levels of c-FLIP. Although c-FLIP is required for activation of caspase-8 at the death-inducing signaling complex,\textsuperscript{29} high levels of c-FLIP act in a dominant-negative manner.\textsuperscript{31} The resulting rate of formation of active caspase-8 is insufficient to initiate the proteolytic cascade. However, caspase-8 cleaves BID, a BH3 containing homolog of EGL-1. Truncated BID aggregates with BAX to form a complex that disrupts mitochondrial membrane and releases cytochrome-c.\textsuperscript{32} Cytochrome-c activates caspase-9, which then initiates the caspase cascade.

Type I and type II terms are generally used in the literature; however, we would like to suggest a third paradigm to emphasize forms of death that are independent of cell membrane death receptors. For example, mitochondria can be injured by free radicals, halogenation, calpains, and p53 activation.\textsuperscript{33–35} Moreover, mitochondrial damage can lead to caspase-dependent or caspase-independent death.\textsuperscript{36,37} Few of these “type III” death pathways have been characterized in macrophages.

### How Do Macrophages Die in the Plaque?

Based on evidence linking Fas to systemic lupus erythematosus (SLE) and SLE to an increased incidence of atheroscler-
The molecules usually implicated in death of plaque macrophages are cytotoxic lipids, but some components of oxidized LDL can promote survival. The first evidence for the oxidized lipid hypothesis appears to be an observation in the 1970s that rabbits fed purified cholesterol developed limited disease, but rabbits fed chow with “brown” cholesterol taken from a laboratory reagent bottle developed large lesions with necrotic cores. Some workers in the field have assumed that the moieties responsible for this promotion of lesion formation are free radicals derived from the oxidized lipids. At least one group, however, reported that oxidized LDL can be antiapoptotic under some circumstances. Such differences may reflect differences in the way oxidized LDL or macrophage are prepared in different laboratories. An understanding of this literature requires very careful attention to the method used to produce “oxidized LDL,” especially the use of copper to oxidize LDL, the extent of oxidation, storage of the product, and dialysis to remove oxidants.

An alternative cytotoxic lipid is cholesterol itself. This hypothesis dates back 2 decades to studies of the ability of lysosomal enzymes to process lipids. The earlier studies suggested that macrophage death occurs because of the inability of the plaque macrophage to de-esterify the massive amounts of cholesterol ester that accumulates in plaque. The cholesterol hypothesis was supported by in vitro studies showing that macrophages, at least in vitro, lack sufficient cholesterol esterase to hydrolyze the cholesterol esters taken up by foam cells. More recent support came from in vivo studies in LDL receptor−/− mice reconstituted with monocytic deficient in acetyl-coenzyme A transferase (ACAT1). Lesion size was increased with large necrotic cores and an increase in TUNEL frequency. More recently, in a similar experiment, Feng et al studied mice with mutation of a protein, Npc1, required for transport of endosomes to the endoplasmic reticulum (ER). Their theory was that cholesterol in the ER causes death by activation of an endosome-dependent ER stress pathway. When mice deficient in Npc1 were crossed with ApoE−/− mice, incidence of TUNEL was decreased, although curiously there was no change in lesion size. The issue of TUNEL is also complicated because TUNEL frequencies, at best, tell us that some death has occurred but do not tell us the rate of cell death. Moreover, apoptotic death may decrease lesion mass or increase it as a result of reaction to cytokines, especially Th2 cytokines, associated with the response to apoptotic cell death. In any case, it seems unavoidable that death of cells in the plaque will increase plaque vulnerability.

Thus, Feng et al have proposed a specific pathway leading from accumulation of free cholesterol to ER injury, followed by activation of an ER-bound caspase, caspase-12. However, cell death by oxidized LDL versus cell death by cholesterol accumulation may use different mechanisms. For example, the frequency of TUNEL-positive macrophages with unesterified cholesterol in vitro in the presence of acetyl-coenzyme A transferase inhibitor is <20%, whereas the level of cell death seen with oxidized LDL approaches 100%. Although in vitro studies implicate caspases in death caused by cholesterol accumulation, mechanisms of death by oxidized...
LDL appear to be caspase-independent. Oxidized LDL kills nearly 100% of cells in the presence of a caspase inhibitor. In contrast, death caused by unesterified cholesterol can be inhibited by caspase inhibitors.40

It is important to point out that very few of the possible mechanisms for death of cells in atherosclerotic lesions have been studied. Toxic agents likely to be present in plaques include oxidized lipids, free radicals, halogenated products, detergents, enzymes, and bacterial products. Moreover, there is evidence for complement activation and p53 activation in plaques.33–35 One study suggests that p53 itself may work by controlling cell surface presentation of Fas.64 Targeted loss of p53 in plaque macrophages has been shown to increase lesion size.65 Furthermore, p53 delivered by adenoviral transfection caused plaque rupture, presumably via death of plaque macrophages.66 The issues with Fas, or other death receptors, are further complicated by potential systemic effects on inflammation that may influence local effects on cell death.67 Finally, the only model with both in vivo and in vitro data are the free cholesterol hypothesis. Clearly, there is a need for both more exploration of different hypotheses and for critical evaluation in animal models.

Caspase Activity Need Not Imply Death
Antibodies to the active form of caspase-3 show that macrophages with activated caspase-3 are present in murine and human plaques, including many cells that are not “dead” by the TUNEL criterion.46 This in vivo observation is supported by studies of macrophage death in vitro. Thioglycolate-elicited mouse peritoneal macrophages spontaneously activate caspase-3 on adherence (Figure 2). Moreover, the activity was confirmed by demonstration of PARP cleavage, the usual marker for caspase activity, in viable macrophages.46 These p17 caspase-3–positive cells can survive and even replicate in long-term passage in vitro. Perhaps even more remarkable, as described, differentiation of human monocytes to macrophages has also been shown to require caspase activation.68 Thus, caspase activation is not necessarily a lethal event in macrophages and may be required for normal physiological functions.

Functions of Active Caspases in Living Cells
Cytoskeletal changes in terminal differentiation are often caspase-dependent (Table). For example, the characteristic enucleation of keratinocytes and lens fiber cells appears to be caspase-mediated.69–72 Caspase inhibitors also suppress the process of nuclear condensation during hematopoiesis73 without concomitant induction of cell death. Incubation with peptidase caspase inhibitors or overexpression of BCL-2 has been shown to block fragmentation of pro-platelets from megakaryocytes,74 and platelet formation is reduced in transgenic mice overexpressing BCL-2, whereas the number of megakaryocytes remains unchanged.74

Whereas thrombopoiesis, erythropoiesis, and keratin formation might be considered as forms frustes of programmed cell death, caspase activity is also required for differentiation of nucleated cells. For example, transient caspase activation is also necessary for differentiation of hematopoietic cells, proliferating lymphocytes, spermatids, myoblasts, and syncytiotrophoblasts, as well as differentiation of monocytes to macrophages.46, 68–71, 73–76 Many of these requirements for caspases in both may be related to proteolysis of cytoskeletal proteins. Apoptotic cells and differentiating cells undergo shape changes. Thus, in myoblasts, deletion of caspase-3 leads to dramatic reduction in myofibers,71 possibly by loss of cell fusion. Similarly, fusion of cytotrophoblasts into syncytiotrophoblasts can be inhibited by downregulation of caspase-8 protein expression, or the inhibition of caspase-8 protein activity.73 Caspases also serve to remove cytoplasm during the process of spermatid individualization from spermatocytes.76 Similar functions might be critical to the ability of macrophage to form extensively modified cell structures, such as cell projections and, possibly, giant cells.

Beyond terminal differentiation and shape changes, caspases appear to play a role in the cell cycle. Direct evidence for caspase involvement in cell cycle has been shown in FADD−/− mice or mice expressing a dominant-negative FADD protein, in which activation-induced proliferation of T cells is impaired.77–79 Caspase-8 is cleaved in nonapoptotic T cells after T-cell receptor stimulation.80 Proliferation of activated T cells is impaired by caspase inhibitors, such as the pan-caspase inhibitor, zVAD-FMK.80 Selective cleavage of caspase substrates has been identified in proliferating T cells. Cell-cycle arrest at the G2/M checkpoint is caused by phosphorylation of CDC2 by an inhibitor of cell-cycle regulator kinase, Weel1. The inhibitory Weel1 is cleaved by active caspases in proliferating T cells. Interestingly, inhibitors of DNase, substrates implicated in cell death, including DFF45 and replication factor, RFC140, remained intact in these proliferating T cells despite the presence of active caspases.

Caspase-Sensitive Kinases: A New Signaling Paradigm
Surprisingly, if caspases are considered only as executioners, many kinases are activated by caspases. In at least one-third of the known examples, caspases activate kinases by removing their autoregulatory domains giving rise to constitutively active molecules and potent inducers of apoptosis.81 Whereas conventional wisdom has been that these activated kinases are proapoptotic, our unpublished observations, as well as those of other groups, show that such activation occurs even in “undead” cells (ie, living cells with activated caspases). For example, several members of the PKC family and MAP kinase pathway are constitutively activated by the separation of regulatory domain from the catalytic domains.82–87 Examples include the p21-activated kinase PAK2, as well as Rho-dependent–kinase 1 (ROCK-1).88–90 Activated PAK2 and ROCK-1 are important for cytoskeletal reorganization and plasma membrane blebbing during apoptosis.88–90 If this subset of caspase substrates is constitutively activated, these cells can quickly respond to environmental cues with amplification of intracellular signals. Relevant to atherosclerosis, inhibition of both normal ROCK-1 and caspase-activated ROCK-1 with Y-27632 has been shown to reduce early atheroma formation.91 Although the caspase-activated form of ROCK-1 has been described in apoptotic cells, our laboratory has found that this form also exists in adherent...
mouse peritoneal macrophages. The short caspase-dependent form of ROCK-1 is constitutively activated, but even more importantly, this form of ROCK-1 is required for blebbing during apoptosis.

Caspase-mediated cleavage of kinases may also direct cell polarity and motility. Although Cdc42 is not a caspase substrate, it interacts with the caspase substrate, Par6-aPKCζ, to regulate glycogen synthase kinase-β and promote centrosome polarization to control the direction of cell protrusion. Furthermore, caspase activation may also serve to autodownregulate inflammatory responses. Caspase-dependent cleavage of cPLA2 has been shown to be Fas-dependent. Thus, Fas–FasL interactions may serve a dual physiological role in monocytes/macrophages. Initially, Fas may regulate differentiation into the macrophage phenotype and subsequently downregulate the inflammatory response via activation of caspases to reduce intracellular cPLA2.

**Speculation: Possible Functions of Active Caspases in Plaque Macrophage**

As already noted, caspase activation of ROCK-1 is necessary for formation of the apoptotic bleb. Apoptotic blebs have several potentially important roles in atherosclerotic plaques. First, they contribute to the phospholipid-rich center of the atheroma affecting, among other things, the fluidity of the necrotic core. Second, apoptotic blebs are believed to form the nidus for hydroxyapatite formation, a critical stage in plaque calcification. Third, apoptotic blebs are likely to contain tissue factor accounting for the covert tissue factor factor found in plasma. Finally, apoptotic blebs are rich in phosphatidylserine. Henson et al have shown that the receptor for phosphatidylserine in an important secretory signal for release of TGF-β. Disruption in the phosphatidylserine receptor is implicated as the mechanism of sustained inflammation in cystic fibrosis. TGF-β is also implicated in plaque fibrosis and in limiting the extent of inflammatory response in the plaque.

As discussed, active caspases are also implicated in macrophage differentiation. Signaling through PKCε is critical for maturation of macrophages. Cleavage of both atypical and novel PKC isoforms has been demonstrated in both apoptotic U937 and HL60 (myelomonocytic cell lines) induced by PMA to become nonapoptotic, adherent cells. Mice deficient in Prkcε (PKCε) are smaller in weight and have frequent Gram-negative bacterial infections caused by impaired host defense responses to lipopolysaccharide (eg, reduced levels of nitric oxide, tumor necrosis factor-α, and IL-1β). Thus, living macrophages with active caspases can cleave Prkcε to amplify lipopolysaccharide-mediated signaling. In addition, mice with disruption of Prkcζ (PKCζ) showed phenotypic alterations in secondary lymphoid organs reminiscent of those in TNF receptor-1 and lymphotixin B receptor–deficient mice. Embryonic fibroblasts lacking Prkcζ were severely impaired in ISβ-dependent transcriptional activity. Again, macrophages and potentially proliferating T cells can amplify NFκB-dependent inflammatory responses by caspase-mediated cleavage of Prkcζ and PKCζ into constitutively active forms. One needs to consider the potential role of active caspases in differentiating of the various types of monocyte-derived cells (eg, dendritic cells, histiocytes, and macrophages) present in the atherosclerotic plaque.

**Summary**

Accumulating evidence suggests that death of plaque macrophages plays a pathogenic role in plaque rupture. Without a necrotic core, the concept of plaque rupture would probably be meaningless. Only 3 specific pathways have been studied. One of these, type 1 Fas-mediated death, has produced conflicting data, perhaps because it has been difficult to distinguish between the role of this receptor in controlling cell death and its role in promoting inflammation. Oxidized LDL, at least in vitro, is an impressive candidate for a critical mediator of cell death in the plaques, but convincing evidence is currently lacking in vivo. The only pathway with good evidence in vivo and in vitro is the role of excess accumulation of free cholesterol. Although evidence implicating Fas, p53, oxidized LDL, and free cholesterol exists, the relative roles of each are not known. Other major candidates to be considered include the toxic effects of specific oxidized lipids, other free radical products in the plaque, phospholipids, and, possibly, bacterial products.

Morphological criteria for apoptosis, including TUNEL, do not prove that a caspase-dependent death process has occurred, or even shed light on specific mechanisms of death. Moreover, caspase activation should not be equated with cell death. Identifying the critical death pathway(s) within the plaque remains an important research objective.

Macrophages in vitro can survive and even replicate with evidence of caspase activity. Immunocytochemical studies suggest, but do not prove, that similar cells with caspase activity exist in plaques. Functions of nonlethal caspases in macrophages are, for that matter, in other cells of the atherosclerotic plaque have only begun to be explored. Before such studies can proceed, however, it may be very important to know more about the role of active caspases in mediating differentiation of monocytes into different cell types within the plaque.

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