

Consequences and Therapeutic Implications of Macrophage Apoptosis in Atherosclerosis

The Importance of Lesion Stage and Phagocytic Efficiency

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Abstract—Macrophage apoptosis occurs throughout all stages of atherosclerosis, yet new findings in vivo suggest that the consequences of this event may be very different in early versus late atherosclerotic lesions. In early lesions, where phagocytic clearance of apoptotic cells appears to be efficient, macrophage apoptosis is associated with diminished lesion cellularity and decreased lesion progression. In late lesions, however, a number of factors may contribute to defective phagocytic clearance of apoptotic macrophages, leading to secondary necrosis of these cells and a proinflammatory response. The cumulative effect of these late lesional events is generation of the necrotic core, which, in concert with proatherogenic effects of residual surviving macrophages, promotes further inflammation, plaque instability, and thrombosis. Thus, the ability or lack thereof of lesional phagocytes to safely clear apoptotic macrophages may be an important determinant of acute atherothrombotic clinical events. Further understanding of the mechanisms involved in macrophage apoptosis and phagocytic clearance might lead to novel therapeutic strategies directed against the progression of advanced plaques. (*Arterioscler Thromb Vasc Biol.* 2005;25:2255-2264.)

Key Words: atherosclerosis ■ macrophage ■ apoptosis ■ phagocytosis ■ inflammation

One of the earliest events in atherosclerosis is the entry of monocytes into focal areas of the arterial subendothelium that have accumulated matrix-retained and often modified lipoproteins.¹⁻⁵ The monocytes differentiate into macrophages, and the macrophages accumulate large amounts of intracellular cholesterol through the ingestion of the subendothelial lipoproteins.^{1,2,6-8} The presence of cholesterol-loaded macrophages in atherosclerotic lesions is a prominent feature throughout the life of the lesion, and these cells have a tremendous impact on lesion progression.⁹⁻¹¹ Therefore, the number of macrophages in lesions is an important measure of atherosclerotic burden. The processes that determine macrophage cellularity in lesions include two factors that promote the accumulation of these cells—monocyte/macrophage entry and macrophage proliferation—and two features that lead to cell depletion—macrophage death and macrophage egress.¹²⁻¹⁶

See cover

This review will address one of these processes, namely, lesional macrophage death. Within this topic, there are many important areas, including evidence that macrophages die in the course of atherosclerosis; inducers and mechanisms of lesional macrophage death; and functional consequences of macrophage death in the setting of atherosclerosis. The first two areas have been covered widely in a number of important articles and comprehensive reviews.^{14,17-26} After a brief

synopsis and update of these two areas, this review will focus on a hypothesis related to the functional consequences of lesional macrophage death. The hypothesis states that (1) the ultimate effect of macrophage death on plaque progression depends on the lesion stage during which macrophage death occurs; and (2) an important factor conferring this dependency on lesion stage is the ability or lack thereof of phagocytes in different stages of atherosclerosis to safely clear dead macrophages. Elements of this hypothesis have been discussed previously by a number of investigators, including Steinberg, Kockx, Libby, and their coworkers,^{20,22,27} and the reader is encouraged to review these articles to gain a historic perspective in this area.

Overview of Macrophage Death in Atherosclerosis

General Principles

In general, cells that die under physiological and pathophysiological circumstances usually go through one of several types of energy-dependent cell death programs referred to as “apoptosis.”^{28,29} These programs are triggered by a number of upstream signaling pathways that act individually or in concert with each other. The best studied of these upstream pathways include those involving cell surface “death receptors,” such as Fas and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, and those generated through perturbation of mitochondrial and endoplas-

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mic reticulum (ER) function. These pathways ultimately converge to activate an array of proteases known as caspases, which carry out many aspects of the death program. Thus, one way that apoptotic death can be identified *in vivo* is by showing evidence of caspase activation through the use of antibodies specific for activated forms of these proteases. One of the eventual consequences of caspase activation—DNA fragmentation—can also be assayed *in vivo* using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). However, some forms of “programmed” cell death appear to be caspase-independent, and TUNEL staining can yield false-positive results.^{14,26,30,31} Therefore, other features are often used to identify apoptotic cells *in vivo*, including externalized phosphatidylserine, as assessed by staining with labeled annexin V, and distinct morphological features of apoptotic cells, notably condensed nuclei. In this context, all four features—caspase activation, DNA fragmentation, annexin V staining, and condensed nuclei—have been observed in variable numbers of macrophages in all stages of atherosclerosis, and the presence of at least two of the features provides good evidence that apoptosis *per se* has occurred.¹⁴ As we shall see, however, the number of detectable apoptotic macrophages is usually very small under conditions of efficient phagocytic clearance.¹⁴ In contrast, when phagocytic clearance is not efficient, one observes not only an increase in apoptotic cells but also the appearance of cells that are swollen and have disrupted membranes. In this case, it is tempting to impugn a different type of death process, often called “necrosis.” However, it is more likely that such cells represent a postapoptotic phase of cell death, referred to as secondary or postapoptotic necrosis, that occurs in the absence of phagocytic clearance of apoptotic cells (below).^{32–35} In this process, the membranes of the apoptotic cells eventually become disrupted, leading to release of intracellular contents, which often induce an inflammatory response.^{34,35}

Inducers of Macrophage Apoptosis in Atherosclerosis

Macrophage death in atherosclerotic lesions is almost certainly multifactorial, and it is likely that at least some of the death inducers are different in early versus late lesions. Despite this complexity and the paucity of *in vivo* data, several proapoptotic factors have been suggested based on cell culture models that attempt to model conditions in atherosclerotic lesions. Examples include high concentrations of oxidized LDL, oxysterols, TNF- α , Fas ligand, nitric oxide, growth factor withdrawal, hypoxia/ATP depletion, and intracellular accumulation of unesterified, or “free,” cholesterol.^{24,26} The oxidized LDL hypothesis of macrophage apoptosis has received widespread attention,^{36,37} and there is one study showing a correlation between antioxidant administration and decreased macrophage apoptosis in rabbit lesions.³⁸ In addition, whereas several studies have shown poor spatial correlation between the presence of oxidized LDL and apoptotic macrophages in advanced lesions,^{23,39} others have shown that oxysterols are rich in advanced lesional necrotic cores.⁴⁰ In terms of antioxidant therapy in humans, which in theory might help prevent acute vascular events by prevent-

ing late lesional macrophage death (below), the results of prospective trials to date have been disappointing.^{41,42} However, these results may be attributable to the use of antioxidants that do not block the specific oxidation reactions that are most relevant to atherosclerosis. Another issue that adds complexity to the oxidized LDL hypothesis of macrophage death is that low concentrations of oxidized LDL can actually promote macrophage survival by activating the Akt cell survival pathway.⁴³

An inducer of macrophage death that is unique to advanced atherosclerotic lesions is the accumulation of large amounts of intracellular free cholesterol (FC), presumably because of defects in both ACAT-mediated cholesterol esterification and cholesterol efflux.⁴⁴ FC loading of ACAT-inhibited, ACAT-deficient, or endotoxin-activated cultured macrophages using lipoproteins internalized by the type A scavenger receptor triggers apoptosis via both Fas and mitochondrial pathways.^{24,45} The intracellular pathways upstream of Fas- and mitochondria-mediated apoptosis involve multiple convergent signals. One signal requires FC trafficking to the ER. This event increases the phospholipid order parameter of, or “stiffens,” the normally fluid ER membrane bilayer and causes dysfunction of ER integral membrane proteins, such as sarcoendoplasmic reticulum ATPase (SERCA).^{46,47} As a result, an ER-based signal transduction pathway called the Unfolded Protein Response (UPR) is activated, and the UPR effector CHOP (GADD153) mediates one of the necessary upstream signals for FC-induced macrophage death.⁴⁷ Two other necessary signals for apoptosis are JNK2 activation, which also results from cholesterol loading of the ER, and engagement of the type A scavenger receptor, or SRA, with a lipoprotein or non-lipoprotein ligand.⁴⁸ Investigations are still underway to fully elucidate the SRA–death pathway and to determine whether the SRA acts in concert with one or more other cell-surface molecules. Regarding *in vivo* relevance of the FC-UPR model, studies have shown that FC-enriched macrophages, as documented by filipin staining, are apoptotic and express CHOP in advanced atherosclerotic lesions.^{49,50} To test causality, advantage was taken of a naturally occurring heterozygous mutation in a cholesterol trafficking protein called NPC1. This heterozygous mutation inhibits trafficking of lipoprotein-derived cholesterol to the ER and thereby blocks FC-induced UPR activation and apoptosis in cultured macrophages.⁴⁷ In advanced aortic root lesions of *ApoE*^{-/-};*Npc1*^{+/-} mice, this subtle cholesterol trafficking alteration caused a marked decrease in lesional necrosis and macrophage apoptosis.⁴⁹

Consequences of Altered Early Lesional Macrophage Apoptosis in Mice

A number of genetic alterations in mouse models of atherosclerosis result in increases or decreases in early lesional macrophage apoptosis, and the overall results of these studies suggest an inverse relationship between early lesional macrophage apoptosis and early lesion area. Van Vlijmen et al⁵¹ reconstituted a mouse model of atherosclerosis (APOE*3-Leiden mice) with bone marrow from mice deficient in the proapoptotic protein p53. As expected, there was a tendency toward decreased macrophage apoptosis in the mice trans-

planted with *p53*^{-/-} versus wild-type bone marrow. The authors also reported no apparent change in the number of proliferating cells. These findings were associated with a 2.3-fold increase in early atherosclerotic lesion area, suggesting that inhibition of early lesional macrophage apoptosis promotes the growth of early atherosclerotic lesions. Of note, a similar *p53*-bone marrow transplantation study in *Ldlr*^{-/-} mice also found an increase in early lesion area, but in this case the effect of macrophage *p53* deficiency was correlated more with an increase in macrophage proliferation than with a decrease in macrophage apoptosis.⁵² More definitive support for the importance of lesional macrophage apoptosis in modulating early atherogenesis came from a recent study in which *Ldlr*^{-/-} mice were transplanted with bone marrow from mice lacking *Bax*, a proapoptotic protein.⁵³ As predicted, early lesional macrophage apoptosis was decreased in the mice receiving *Bax*^{-/-} bone marrow versus wild-type marrow, and this was associated with an increase in early aortic root lesion cellularity and area.⁵³ The data from this study and the aforementioned study of van Vlijmen et al suggest that macrophage apoptosis is normally occurring in early atherosclerosis, perhaps via one or more pathways that involve *p53* and *Bax*, and that apoptosis modulates early lesion size and cellularity. In another study that complements these findings, Arai et al⁵⁴ found that absence of an LXR-inducible macrophage survival protein known as AIM (apoptosis inhibitor expressed by macrophages; also known as Sp α or API6) renders macrophages highly susceptible to oxidized LDL-induced death in vitro and reduces early aortic lesion area in *Ldlr*^{-/-} mice. In summary, macrophage apoptosis in early foam cell lesions in mice appears to limit lesion cellularity and progression. In the following sections, this principle will be linked to the hypothesis that phagocytic clearance of apoptotic macrophages in early atherosclerotic lesions is highly efficient.

Phagocytic Clearance of Apoptotic Cells

General Principles

Extensive in vivo investigation has shown that the most important influence on the steady-state number of apoptotic cells and on the physiological consequences of apoptosis is phagocytic clearance of apoptotic cells.^{32,33} During the course of apoptosis, cells lose cell-surface proteins that normally inhibit recognition by phagocytes and express cell-surface and secreted molecules that promote such interaction. In the normal course of events, apoptotic cells are very rapidly and efficiently recognized and internalized by phagocytes. This function can be carried out by both "professional" phagocytes, ie, macrophages and neutrophils, as well by a number of other cell types, such as epithelial cells.^{32,33} The process is so efficient, rapid, and high-capacity, that when one observes more than a few percent of apoptotic cells in tissues, it usually means some defect in phagocytic recognition or uptake.

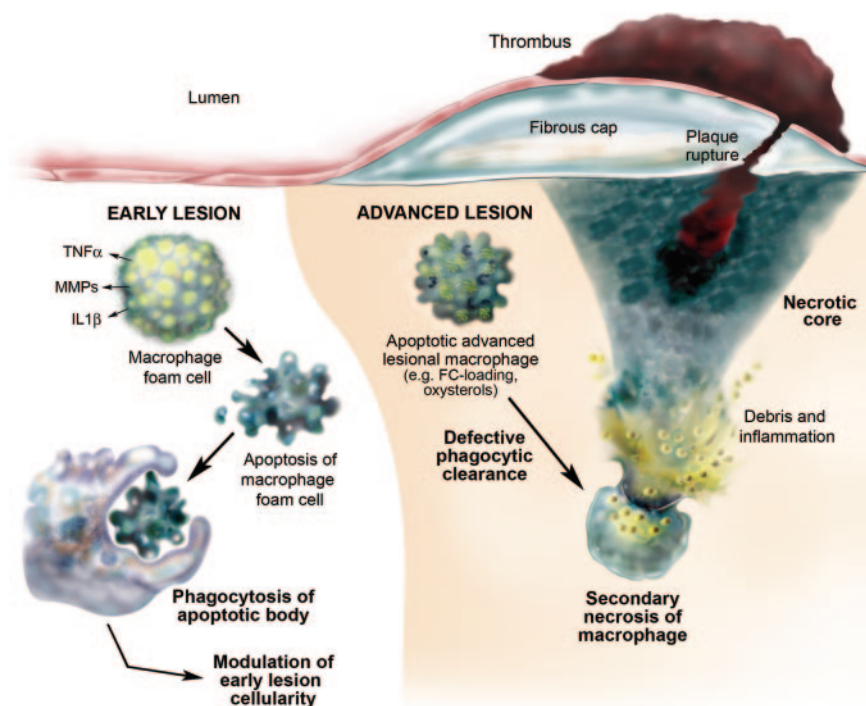
The interaction of phagocytes with apoptotic cells leads to a number of important consequences. Most notably, there is a suppression of inflammatory signaling and activation of antiinflammatory pathways.^{32,33} In contrast, the interaction of phagocytes with necrotic cells often results in an inflamma-

tory response.⁵⁵ In a related area with potential relevance to atherosclerosis, dendritic cells that have phagocytosed apoptotic cells can present apoptotic cell-associated antigens to T cells.⁵⁶ This process can result in either T cell activation or tolerance, depending on the types of phagocyte receptors involved in apoptotic cell uptake and the overall milieu in which these events are occurring.⁵⁶ For example, in certain settings, dendritic cell phagocytosis of necrotic cells but not apoptotic cells results in T cell activation.⁵⁷ Finally, cell culture studies have suggested that phagocytosis of apoptotic cells by macrophages can alter recruitment, survival, and proliferation of the macrophages themselves as well as neighboring cells. For example, phagocytic clearance of apoptotic cells leads to transforming growth factor (TGF)- β secretion, which in turn can inhibit monocyte recruitment into inflammatory lesions.⁵⁸ In addition, phagocytosis of apoptotic cells promotes phagocyte survival and suppresses phagocyte proliferation.⁵⁹ Moreover, phagocytosis of apoptotic T cells by macrophages results in VEGF secretion, which can promote endothelial cell proliferation.⁶⁰ If these events occur in atherosclerotic lesions, they might have important effects on lesion cellularity, endothelial repair, and neovascularization.

With this background, one can begin to appreciate the potential consequences of perturbed phagocytic clearance of apoptotic cells. The apoptotic cells become secondarily necrotic, which can cause direct tissue damage from released intracellular proteases and other noxious material from the dead cells. Some of this debris may also promote coagulation and thrombosis (below). Most importantly, a robust inflammatory response may result by direct triggering of proinflammatory responses and inhibition of antiinflammatory responses in the phagocytes. If dendritic cells are present, uptake of the necrotic cells by these phagocytes might further promote inflammation through T cell activation. Finally, defective phagocytosis may promote further monocyte recruitment, decreased survival of remaining competent phagocytes, and perturbation of endothelial repair processes. As we shall see below, many of these consequences may be highly relevant to advanced atherosclerosis.

Apoptotic Cell Clearance in Early Atherosclerotic Lesions

The in vivo data discussed above showing that decreasing early lesional macrophage apoptosis in mice increases lesion area implies that the normal amount of macrophage apoptosis occurring in these lesions limits lesion area. In order for this to occur, it is almost certain that phagocytic clearance of apoptotic macrophages in early lesions is very efficient and physiologically beneficial. Indeed, the data of Arai et al⁵⁴ cited above suggest that the capacity of early lesional phagocytic clearance is quite high, because lesion area was further decreased in the face of an artificial increase in early lesional macrophage apoptosis (Figure, left). Consistent with this concept, cell-culture studies have shown that cholesteryl ester-loaded macrophages, the most prominent type of phagocyte in early lesions, can effectively recognize and ingest apoptotic macrophages⁶¹ (unpublished data, 2005). In addition, withdrawal of an atherogenic diet in rabbits with established foam cell lesions leads to a gradual disappearance



Model of how lesion stage and phagocytic efficiency might determine the consequences of macrophage apoptosis in atherosclerosis. In early lesions (left), macrophage foam cells undergo apoptosis and are then rapidly and safely cleared by neighboring phagocytic macrophages. Because living foam cells play an overall pro-atherogenic role by secreting cytokines and other molecules, the net effect of early lesional macrophage apoptosis is modulation of lesion cellularity and decreased lesion progression. In late lesions (right), macrophages also undergo apoptosis, albeit by mechanisms that are probably unique to these lesions, such as free cholesterol (FC) loading and exposure to oxysterols. In this setting, however, phagocytic clearance is not efficient, and secondary necrosis of the macrophages ensues. This event leads to the generation of the necrotic core, which promotes inflammation, plaque instability, and acute lesional thrombosis. Not shown here is the likely important role that residual surviving macrophages play in promoting the progression of advanced lesions. See text for details.

of apoptotic macrophages, which may further indicate efficient phagocytic clearance of apoptotic macrophages in these relatively early lesions.⁶² Thus, effective phagocytosis, together with evidence indicating that living macrophages promote plaque progression,^{9–11} likely explain why increasing early lesional macrophage apoptosis has antiatherogenic consequences, and vice versa.²⁰

Apoptotic Cell Clearance in Advanced Atherosclerotic Lesions

Apoptotic macrophages are more numerous in advanced versus early atherosclerotic lesions, suggesting that phagocytic clearance in advanced lesions is defective (Figure, right).^{14,20,27} Direct support for this idea was provided recently by Schrijvers et al,⁶¹ who showed that human carotid atherosclerotic lesions contained a substantial number of apoptotic cells that were not engulfed by phagocytes. In contrast, most of the apoptotic cells observed in human tonsillar tissue were inside phagocytes. In addition, a number of studies have shown that late lesional apoptotic macrophages are more numerous in areas surrounding the necrotic core of these late lesions.^{17,18,23,63} Because the necrotic core is made up primarily of dead macrophages and are rich in inflammatory cytokines, these observations are consistent with the prediction that defective phagocytosis would lead to postapoptotic macrophage necrosis and a heightened state of inflammation (above).^{64–66} Indeed, several recent studies have begun to establish a relationship between defective clearance of apoptotic macrophages and enhanced inflammation in atherosclerosis, as has been found previously in other diseases with a chronic inflammatory component.^{67,68} For example, Khan et al⁶⁹ demonstrated that inhibition of apoptotic body clearance by oxidized LDL (see below) in a cell culture model led to enhanced secretion of inflammatory

cytokines, including interleukin (IL)-6. Moreover, Grainger et al⁵⁸ found that defective clearance of apoptotic cells in *Apoe*^{−/−} mice was associated with elevated levels of TNF- α and other inflammatory markers in vivo. In a study designed to model events in advanced lesions, we recently found that the robust production of TNF- α and IL-6 by FC-loaded macrophages⁷⁰ could be markedly suppressed by coculture with competent macrophage phagocytes (unpublished data, 2005).

In addition to promoting inflammation, the accumulation of noncleared apoptotic cells and postapoptotic necrotic cells likely has a number of other consequences that would be predicted to promote late lesion progression: (1) noncleared apoptotic cells can be a source of tissue factor,^{20,71,72} a procoagulant molecule thought to play an important role in coagulation and thrombosis associated with advanced plaques;⁷³ (2) necrotic core formation, resulting from postapoptotic macrophage necrosis, is associated with the accumulation of lysophosphatidic acid derivatives, which enhance platelet aggregation⁷⁴ and may prevent the emigration of macrophages from atherosclerotic lesions;¹⁶ (3) inflammatory factors in the necrotic core may induce surrounding macrophages to secrete matrix-degrading proteases in atheromata,⁷⁵ and it is also possible that proteases released directly from necrotic macrophages could contribute to plaque instability; (4) as alluded to above, phagocytes that fail to internalize apoptotic cells may be more susceptible to apoptosis themselves,⁵⁹ leading to further depletion of the phagocyte pool in lesions and amplification of the detrimental events outlined above; and (5) necrotic macrophages release a potent angiogenic factor, thymidine phosphorylase, which is abundant in the necrotic core debris of advanced atherosclerotic lesions.⁷⁶ In this context, Moulton et al⁷⁷ have provided evidence that plaque neovascularization promotes lesion progression.

Therefore, defective phagocytic clearance of apoptotic macrophages in advanced plaques could promote a number of processes that are thought to be important in plaque disruption and acute atherothrombotic vascular occlusion.^{20,27}

Possible Mechanisms of Defective Phagocytic Clearance of Apoptotic Macrophages in Advanced Atherosclerotic Lesions

An interesting hypothesis to explain why phagocytic clearance of apoptotic cells is inefficient in advanced atheromata has been developed over the last 10 years by Steinberg, Witztum, Miller, and colleagues. These investigators have shown that oxidized molecules known to be present in atherosclerotic lesions, particularly in necrotic cores, inhibit the uptake of apoptotic cells by phagocytes.⁷⁸ The proposed mechanism involves competitive inhibition of receptors, because certain phagocyte receptors for apoptotic cells, such as the type A scavenger receptor, CD36, and CD68, recognize these oxidized molecules. In addition, naturally occurring antibodies to oxidized LDL in atherosclerotic lesions, by binding phagocyte ligands on apoptotic macrophages, may block recognition and ingestion by phagocytes.⁷⁹ Another mechanism related to oxidation is based on cell-culture data showing that a minimally oxidized form of LDL present in lesions inhibits phagocytosis of apoptotic cells by altering actin polymerization in the phagocytes.^{80,81} Possible evidence for the interference of phagocytic clearance by oxidized lipids *in vivo* was obtained by Walsh and colleagues,⁸² who found that mice with increased apoptosis had defective clearance of apoptotic cells in the face of hypercholesterolemia, which promotes the formation of oxidized lipids, or by infusion of lysophosphatidylcholine, a component of oxidized LDL. Nonetheless, the fact remains that clinical trials in humans with antioxidants have not been successful in preventing coronary artery disease, although, as stated previously, it is possible that more encouraging results will be obtained in the future through the use of drugs that are targeted to the specific oxidation reactions in atherosclerosis. In this context, it is interesting to note that certain antioxidants may actually *inhibit* recognition of apoptotic cells by phagocytes by inhibiting oxidation of externalized phosphatidylserine.⁸³

Another mechanism of defective phagocytosis was suggested by Schrijvers et al,⁶¹ who showed that cultured macrophages loaded with indigestible beads or subjected to oxidant stress had a compromised ability to ingest apoptotic cells. The finding with indigestible beads may be related to a hypothesis suggested by Kockx that ingestion of erythrocytes by lesional macrophages renders them incompetent to phagocytose apoptotic cells in lesions.²⁰ A potential mechanism relevant to the FC model of lesional macrophage apoptosis was suggested by Su et al, who found that FC-loading of macrophages resulted in downregulation of milk fat globule-EGF factor 8, a molecule involved in the phagocytosis of apoptotic cells.^{84,85} Finally, given that the most abundant phagocytes in advanced lesions are macrophages themselves, macrophage death would be expected to deplete the pool of phagocytes in these lesions and therefore amplify the accumulation of dead macrophages (above).

An additional issue that deserves mention is macrophage heterogeneity within lesions. Studies have shown that certain cell-surface markers are expressed differently on macrophages close to the endothelial layer versus those in the deeper aspects of plaques.⁸⁶ These differences may reflect different stages of monocyte-to-macrophage differentiation, different degrees of lipid loading, and/or influences of focal extracellular molecules.^{86,87} Given that some of the differential cell-surface markers can be involved in the uptake of apoptotic cells⁸⁷ and that phagocytosis efficiency changes with stage of differentiation,⁸⁸ the relative proportion of phagocytic-defective subpopulations of macrophages in lesions may affect the clearance of apoptotic cells and the sites within plaques where lesional necrosis occurs.

Future studies may reveal additional mechanisms based on other diseases with defective phagocytosis. For example, there is defective apoptotic cell clearance in cystic fibrosis patients with inflammatory bronchiectasis, and one proposed mechanism is the cleavage of macrophage phagocyte receptors by neutrophil elastase.⁶⁸ In this regard, Libby and colleagues demonstrated the presence of abundant neutrophil elastase activity in macrophage-rich areas of advanced human atherosclerotic lesions.⁸⁹ Additionally, there are situations in normal physiology in which phagocytic clearance of apoptotic cells is inhibited,⁹⁰ and elucidation of the inhibitory mechanisms involved in these processes may suggest new hypotheses for defective apoptotic clearance in advanced atherosclerosis. For example, the clearance of apoptotic cells by macrophages is blocked by the long pentraxin PTX3,⁹¹ and this molecule has been found to be expressed in advanced human atherosclerotic lesions.⁹²

Therapeutic Implications

General Principles

The therapies currently available for atherothrombotic disease are those that modify risk factors, such as hypercholesterolemia, smoking, and diabetes, and those that suppress platelet aggregation. Although highly successful, the population at risk even after these interventions is very large. In the future, complementary therapeutic approaches directed at the level of the arterial wall, while currently challenging and speculative, may be required to substantially further reduce the incidence of cardiovascular disease. In this context, the principles put forth in this review may provide ideas for future arterial-wall strategies, as described below.

There are a few caveats that should be mentioned at the outset. First, the heterogeneity of lesion stage at any given point in time and the heterogeneity of macrophage populations in lesions (above) renders some, though not all, of the proposed approaches described below particularly challenging. Second, the ability to deliver drugs to the appropriate areas of lesions at the critical time for a necessary duration adds an additional technical challenge, although the advent of stent-delivered drugs may provide an approach to overcome this obstacle. Finally, because the topic of this review is apoptosis and phagocytic clearance of apoptotic cells, the section on therapy below is focused on these processes. However, strategies to limit macrophage cellularity by pre-

venting monocyte entry and macrophage proliferation and by enhancing macrophage emigration should also be considered in a more global view of macrophage-directed arterial wall therapies.

Enhancing Macrophage Apoptosis in Early Atherosclerotic Lesions

As summarized previously, early lesional macrophage apoptosis appears to have an overall beneficial effect on atherosclerosis, which is largely because of the ability of phagocytes in these early lesions to efficiently and safely clear the dead macrophages. Therefore, clever proapoptotic strategies similar to those being investigated for cancer therapy might be worth considering.^{25,93,94} However, a therapeutic strategy based on this concept in humans is likely to encounter a number of problems. First, the intervention would have to be focal, sustained, and cell-specific. Systemic, nonspecific enhancement of apoptosis is obviously not desirable. Even if limited to macrophages, systemic macrophage death would breach host defenses. Assuming that a proapoptotic agent could be delivered locally and in a sustained manner, cell specificity would be required to avoid the potentially damaging effects of endothelial and smooth muscle cell death. The latter issue may be particularly important in view of the theory that lesional smooth muscle cells promote protective fibrous cap formation.²² Macrophage-specific delivery systems, including those involving macrophage-targeted photodynamic therapy, may have a role in this regard.^{95,96} Finally, in mouse models, genetic manipulations that enhance macrophage apoptosis are present at birth or, in the case of bone marrow transplantation, early in atherogenesis.^{51,54} These interventions can therefore affect all atherosclerotic lesions from the beginning. In contrast, interventions in humans would be carried out at a time when both early and advanced lesions are present. Given that macrophage apoptosis in advanced lesions may be detrimental (above), one might have to conceive of ways to selectively promote apoptosis in early lesional versus late lesional macrophages. The approach to such a task is not obvious and would certainly require more knowledge of how early lesional versus late lesional macrophages die (below).

Preventing Macrophage Apoptosis in Advanced Lesions

A therapeutic strategy targeted to the problem of late lesional necrosis would be to inhibit advanced lesional macrophage death. The method of inhibiting macrophage death would have to be selective for advanced lesions to avoid possible proatherogenic effects of inhibiting macrophage death in coexisting early lesions (above). Given that macrophages in advanced lesions probably die by mechanisms that are different from those in early lesions (above), such a strategy may be possible. For example, the accumulation large amounts of cytotoxic FC by macrophages occurs only after lesions have progressed to advanced stages,⁴⁴ and knowledge of the mechanism of FC-induced macrophage death provides a possible selective strategy. In particular, inhibiting the trafficking of lipoprotein-derived cholesterol to the endoplasmic reticulum selectively blocked FC-induced death in cultured

macrophages and decreased macrophage death and necrosis in advanced atherosclerotic lesions *in vivo*.^{47,49} Nonetheless, despite the "success" of this particular model, strategies designed to inhibit a specific inducer of macrophage death may not be effective enough given that late lesional macrophage death is almost certainly multifactorial. A second potential problem is that the increased number of surviving macrophages resulting from this strategy might promote lesion progression by other means, such as by secreting proatherothrombotic molecules.^{97,98}

Enhancing Phagocytic Clearance of Apoptotic Macrophages

An alternative strategy to that mentioned above would be to therapeutically enhance phagocytic clearance of apoptotic cells. To the extent that apoptotic clearance is generally a beneficial process throughout the body, albeit with a few exceptions,⁹⁰ such an intervention would probably be benign even if it were systemic. In the case of early atherosclerotic lesions, this intervention might be neutral or beneficial, depending on whether phagocytosis in these lesions was already maximal or amenable to further enhancement. In advanced lesions, the potential benefits are obvious based on the concepts put forth in this review. Moreover, assuming that the proclearance intervention would not alter the fundamental property of phagocytes to selectively recognize and ingest only apoptotic cells,^{32,33} the theoretical danger mentioned above regarding removal of viable endothelial cells and smooth muscle cells should not be a problem. Therefore, the issue becomes one of implementation.

One possible way to achieve the goal of enhanced phagocytic clearance of late lesional apoptotic macrophages would be to eliminate putative antiphagocytic factors in these lesions. This approach would require a more thorough understanding of these factors (above) and the creation of novel interventions to eliminate them in a safe manner. To the extent that oxidation may be an important anticlearance factor, advances over existing, unsuccessful antioxidant approaches would be needed (above). Another strategy would be to administer agents that directly promote phagocytic clearance of apoptotic cells. Godson and colleagues have begun to explore this strategy as a potential therapy for chronic inflammatory and autoimmune diseases, where defective clearance of apoptotic cells has been implicated in disease progression.⁹⁹ For example, glucocorticoids are known to stimulate phagocytic clearance of apoptotic cells,⁹⁹ but the serious adverse effects of glucocorticoid therapy render this an impractical approach. However, one of the proteins induced by glucocorticoids, annexin-1, stimulates noninflammatory clearance of apoptotic cells by macrophages via an effect on the macrophage actin cytoskeleton.¹⁰⁰ Of interest, this is the same area of cellular function that is perturbed by minimally oxidized LDL, an anticlearance factor that may have relevance to atherosclerosis (above). The development of annexin-1-derived peptides that have similar phagocyte enhancing effects¹⁰⁰ may provide the basis of a future therapeutic intervention.

A second class of molecules that promotes phagocytic clearance of apoptotic cells is the lipoxin family.¹⁰¹ Lipoxins

are eicosanoids that are produced in inflammatory responses through multicellular reactions involving the lipoxygenase pathway.¹⁰² Of interest, lipoxins may be naturally formed during atherosclerosis.¹⁰² Godson et al¹⁰¹ have shown that several members of this family, including LXA4, LXB4, aspirin-triggered 15-epi-LXB4, and their stable analogues stimulate macrophages to phagocytose apoptotic neutrophils. The signaling pathway appears to involve the lipoxin receptor and inhibition of protein kinase A, and a similar clearance-enhancing effect was produced by incubating macrophages with the protein kinase A inhibitor Rp-cAMP.¹⁰¹ Interestingly, the ability of annexin-1 to enhance phagocytic clearance (above) was blocked by a lipoxin receptor antagonist,¹⁰⁰ raising the possibility of a common signaling pathway for these two types of clearance enhancers. Most importantly, Godson and colleagues¹⁰³ have begun to provide relevance in vivo by showing that lipoxins stimulated the phagocytic clearance of neutrophils in thioglycollate-induced peritonitis. Indeed, lipoxins have been shown to have a beneficial effect in a number of experimental inflammatory disease models, but how much of this benefit is due to enhancement of apoptotic cell clearance versus other antiinflammatory effects of these molecules¹⁰⁴ remains to be determined.

Finally, as mentioned above, apolipoprotein E deficiency results in defective phagocytosis of apoptotic cells and increased inflammation in vivo.⁵⁸ Most importantly, incubation of *ApoE*^{-/-} macrophages with exogenous apolipoprotein E3 not only corrected that phagocytosis defect but rendered these cells even more competent than wild-type macrophages.⁵⁸ Therefore, if apolipoprotein E in late lesional macrophages was a rate-limiting factor, administration of the protein or a mimetic peptide locally or a D-amino acid peptide mimetic orally may form the basis of a proclearance therapy.

In summary, the emergence of strategies to enhance the safe phagocytic clearance of apoptotic cells in chronic inflammatory conditions, although still at a very early stage of development and thus speculative at this time, may provide the basis for a novel therapeutic strategy to prevent the progression of advanced atherosclerotic lesions.

Summary and Conclusions

Macrophage apoptosis occurs at all stages of atherosclerosis and is likely triggered by a combination of factors and conditions that almost certainly differ depending on lesion stage (Figure). The consequences of macrophage apoptosis on lesion progression depend to a large extent on how efficiently the apoptotic cells are cleared by intimal phagocytes, which themselves are mostly macrophages. Early atherosclerotic lesions are filled with cholesterol-loaded yet otherwise functioning phagocytes, and both cell-culture and in vivo studies indicate that these cells can efficiently clear the apoptotic macrophages. The result is a decreased number of proatherogenic macrophages without an increase in necrosis or inflammation, leading to an overall antiatherogenic effect. In striking contrast, in vivo observations and cell-culture studies provide support for the hypothesis that clearance of apoptotic macrophages in advanced lesions is defective. The consequences of this defect are likely to be

substantial, because it promotes three processes—necrosis, inflammation, and thrombosis—that are thought to play an important role in plaque disruption and its acute clinical consequences.

Although this hypothesis fits the limited data thus far, it is important to emphasize that more proof is needed. For example, it is almost certain that residual surviving macrophages in advanced lesions also contribute to plaque vulnerability by secreting matrix metalloproteinases and other proatherothrombotic molecules.^{97,98} These cells may also induce death in collagen-producing smooth muscle cells, thus further promoting plaque instability.¹⁰⁵ Thus, the combination of late lesional macrophage death sans phagocytosis, which leads to necrotic core formation, inflammation, and thrombosis, and residual macrophage survival, which maintains cells that can actively promote lesion vulnerability, may be the most important factors in promoting plaque disruption and acute clinical events. A thorough understanding of the mechanisms and consequences involved in these processes will be critical for translating these concepts into novel therapeutic strategies. The most convincing data on stage-specific consequences of macrophage death will come from innovative models in which lesional macrophage apoptosis can be triggered in a temporal and regulated manner^{106,107} to coincide with early versus late atherosclerosis. Other models in which phagocytic clearance of apoptotic macrophages is altered¹⁰³ will also be critical to evaluate the hypotheses discussed herein. In addition to providing important information about the pathophysiology of atherosclerosis, the data from these studies might form the basis of novel therapeutic approaches to combat advanced lesion progression.

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