Current Perspective on the Role of Apoptosis in Atherothrombotic Disease

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Abstract—Thrombus formation on a disrupted atherosclerotic plaque is a threatening event that leads to vessel occlusion and acute ischemia. In this current perspective, we present evidence for apoptosis as a major determinant of the thrombogenicity of the plaque lipid core and a potential contributor to plaque erosion and associated thrombosis. Moreover, apoptosis may directly affect blood thrombogenicity through the release of apoptotic cells and microparticles into the bloodstream. (Circ Res. 2001;88:998-1003.)

Key Words: apoptosis □ thrombosis □ atherosclerosis □ acute coronary syndromes

Acute ischemic syndromes (ie, unstable angina, myocardial infarction, and stroke) are severe clinical manifestations of atherosclerotic disease and account for most of the morbidity and mortality of atherosclerosis. They are primarily related to occlusion of the main vessel lumen by a thrombus formed on the contact of disrupted atherosclerotic plaques. The thrombus, or part of it, eventually embolizes into the microvasculature, leading to disseminated microvascular obstruction. Whatever the mechanism of plaque disruption (ie, deep rupture or superficial erosion), thrombus formation is the most threatening event. Therefore, identification of the pathophysiological mechanisms involved in plaque thrombogenicity is critical to our understanding of these clinical manifestations and may open the way to the elaboration of novel therapeutic strategies to prevent these serious events. The potential beneficial or detrimental roles of apoptotic death in plaque development have been recently reviewed in the literature. In this study, we present evidence for an etiological role of apoptosis as a contributor to thrombus formation and embellishment leading to acute ischemic syndromes.

Occurrence of Apoptotic Death in Atherosclerotic Disease

Apoptosis is a major event occurring during atherosclerotic plaque development. All cell types are involved, with a high predominance of apoptotic macrophages. The distribution of apoptosis is heterogeneous within the plaque, being more frequent in regions rich in inflammatory cells and proinflammatory cytokines and much less abundant in regions characterized by a significant production of anti-inflammatory cytokines. This close association between apoptosis and inflammation suggests that regions of plaque disruption, which are known to be inflammatory, may expose a high percentage of apoptotic cells and apoptotic debris to the circulating blood.

Apoptosis is widely recognized as a clean death. Apoptotic cells and bodies are recognized by adjacent professional and nonprofessional phagocytes and are rapidly removed from the tissue without inducing an inflammatory response. However, this dogma was recently challenged by studies showing Fas-mediated activation of several proinflammatory genes during the apoptotic process. In addition, with regard to atherosclerotic plaques, recent in vitro studies suggest that removal of apoptotic cells may be inefficient in such a complex tissue. Indeed, oxidized phospholipids, as well as antibodies directed against them, which are abundant in advanced plaques, affect recognition of apoptotic or damaged cells by macrophages. Therefore, it is likely that the capacities of clearance of apoptotic cells are reduced in foam macrophages that are in an oxidation-rich environment. This would lead to the persistence within the plaque of apoptotic bodies with potentially high immunogenic properties. Moreover, in some circumstances apoptotic cells are prone to undergo secondary necrosis, and this may lead to accumulation of extracellular lipids and to perpetuation of the inflammatory response. Besides these potential immunoinflammatory effects, we believe that one of the major roles of apoptosis in atherosclerosis is related to its high procoagulant potential.

Procoagulant Potential of Apoptotic Cells and Microparticles

The occurrence of phosphatidylserine (PS) in the exoplasmic leafet of the plasma membrane is considered one of the hallmarks of cells undergoing apoptosis and more generally constitutes one of the determinants for the phagocytosis of apoptotic cells to be rapidly cleared. Once accessible, PS
acquires a procoagulant potential, owing to its ability to promote the surface assembly and the catalytic efficiency of the characteristic enzyme complexes of the blood coagulation cascade, including the tissue factor (TF)/factor VIIa complex. This parallels PS externalization in activated platelets, which constitutes the basis of the platelet coagulant response.

PS exposure at the surface of apoptotic lymphocytic, monocytic, smooth muscle, or endothelial cells can induce procoagulant responses. Flynn et al have shown a thrombin-generating potential of vascular smooth muscle cells derived from human coronary atherosclerotic plaques that undergo apoptosis spontaneously in vitro. The thrombin-generating capacity was secondary to PS exposure. Other studies suggest that endothelial cells that cover the atherosclerotic plaques may also become procoagulant after apoptosis induction. Bombeli et al have shown that apoptotic human umbilical vein endothelial cells (HUVECs) become procoagulant by increased expression of PS and loss of anticoagulant membrane components, including thrombomodulin, heparan sulfates, and TF pathway inhibitor. Moreover, these authors reported a marked increase in the binding of nonactivated platelet to apoptotic HUVECs. Taken together, these data provide evidence that cells undergoing apoptosis, whatever their origin, may contribute to thrombotic events.

Interestingly, PS-dependent procoagulant activities are also detectable in the supernatant of various apoptotic or stimulated cells. The supernatant procoagulant activities are clearly related to the degree of apoptosis in cultured cells and are accounted for by the release of microparticles, probably stemming from surface blebs of apoptotic cells. These procoagulant activities are not different from those of microparticles shed from activated, nonapoptotic cells or platelets. These in vitro observations strongly suggest that apoptotic cells and microparticles may play an important role in the initiation or perpetuation of thrombotic processes.

Apoptosis as an Important Determinant of Plaque Thrombogenicity

Thrombogenicity of the Ruptured Atherosclerotic Plaque

Pathological and functional studies have identified cellular and extracellular TF as a major determinant of the thrombogenicity of the plaque lipid core. TF is a 47-kDa transmembrane glycoprotein that initiates blood coagulation by binding the coagulation factor VII and its activated form (factor VIIa) to form a high-affinity complex. This binary complex proteolytically activates factors IX and X, which in turn leads to thrombin generation. Thrombin generation will, in turn, activate platelets, induce TF, and activate coagulation factor IX-X pathway. The overlying thrombus is extremely thrombogenic, and in the vast majority of cases (85%), it is rich in fibrin. TF activity is significantly higher in plaque from patients with unstable angina and myocardial infarction than in that from patients with stable angina. TF activity is mainly localized in the lipid-rich atheromatous core and is directly related to the thrombogenicity of the plaque material. Moreover, specific inhibition of vascular TF by the use of recombinant TF pathway inhibitor is associated with a significant reduction of acute thrombus formation in lipid-rich plaques. These findings underscore the critical role of the extrinsic coagulation pathway in the generation of acute ischemic syndromes but do not provide any mechanistic explanation for the enhanced TF activity of the plaque lipid core.

TF is operational on the surface of cell membranes, and it is known that its activity is highly dependent on the presence of anionic phospholipids, chiefly PS. Because apoptosis is associated with significant PS exposure on the cell surface and leads to the shedding of PS-containing membrane microparticles, we hypothesized that apoptosis may be directly responsible for TF activation within the plaque. By use of immunohistochemical techniques, we found a colocalization between TF expression (cellular and extracellular) and apoptotic death, particularly in the lipid core, suggesting that TF may be released in apoptotic microparticles during cell death. This was confirmed by isolating shed membrane microparticles from the lipid core of plaques. Quantification of the microparticles by use of a prothrombinase assay revealed significantly higher levels of PS- and TF-containing microparticles in plaque supernatants compared with extracts of normal arteries. In addition, we found that these plaque-derived apoptotic microparticles account for almost all TF activity of the plaque extracts, indicating a direct causal relationship between their presence and TF activity. Although we do not exclude a contribution from other cell types present in the plaque, we have found that most of the microparticles originated from macrophages and lymphocytes that are known to be abundant at sites of plaque rupture. These results suggest that shed membrane apoptotic microparticles play a major role in the initiation of the coagulation cascade after plaque rupture and exposure of the lipid core to the circulating blood. The recent findings that macrophage apoptotic death is significantly increased at sites of plaque rupture and thrombosis in patients with sudden coronary death and that apoptosis is significantly increased in unstable versus stable human plaques strongly support our hypothesis.

Thrombogenicity of the Eroded Atherosclerotic Plaque

Plaque rupture of a thin fibrous cap overlying a lipid core is not necessarily the only final common pathway in the formation of coronary thrombi. Virmani and colleagues recently reported several consistent studies showing that plaque erosion without rupture is an important predisposing substrate for acute coronary syndromes (ACSs) and sudden cardiac death. Although risk factors predisposing to plaque erosion have been identified, the cellular and molecular mechanisms responsible for this process remain unknown. We hypothesized that apoptosis of luminal endothelial cells may be one of the mechanisms leading to erosion and thrombosis.

Vascular endothelial cells are continuously exposed to a range of hemodynamic forces that have a great impact on their cellular structure and function. HUVECs cultured under static conditions undergo a basal low level of apoptosis,
whereas exposure to flow inhibits the apoptotic process.\textsuperscript{,38} Using carotid human atherosclerotic plaques, we recently found that blood flow exerts a direct influence on endothelial cell survival in human atherosclerosis.\textsuperscript{,39} Analysis of longitudinal plaque sections revealed the presence of luminal endothelial cell apoptosis in 60% of plaques examined. Interestingly, luminal endothelial cell apoptosis in these nonruptured plaques occurred preferentially in the downstream parts of the plaques where low shear prevails in comparison with the upstream parts.\textsuperscript{,39} The increase in apoptosis was not balanced by an increase in cell proliferation, suggesting that relatively large areas of endothelial erosion may occur in the distal part of atherosclerotic plaques as a consequence of endothelial apoptosis. This is supported by ultrastructural studies showing frequent endothelial denudation\textsuperscript{,40} or accelerated endothelial senescence in regions of disturbed flow located downstream from the stenosis.\textsuperscript{,41} Given the high procoagulant and proadhesive potentials of apoptotic endothelial cells (see above) and the propensity of denuded vessel segments to increased vasospasm and platelet aggregation, primary endothelial cell apoptosis and secondary denudation in regions of low or disturbed flow may lead to lumen thrombosis favoring plaque progression or occurrence of ACS. Interestingly, Ledru et al\textsuperscript{,42} recently examined geometric features of coronary artery lesions favoring acute occlusion and myocardial infarction. They found that a steep outflow angle of a stenosis, which is a feature characteristic of disturbed flow downstream from the stenosis, is an independent predictor of infarction at 3-year follow-up.

Farb et al\textsuperscript{,46} found that the amount of fibrin and platelets within thrombi formed on the contact of eroded plaques was similar to that in thrombi formed on the contact of ruptured plaques. Nearly 40% of thrombi formed on eroded plaques were predominantly composed of fibrin, which means that early fibrin deposition is present in the absence of plaque rupture. This finding is intriguing, because thrombi that usually develop on subendothelial collagen after superficial endothelial denudation are known to be predominantly, if not exclusively, composed of platelets. Therefore, we believe, like others,\textsuperscript{,43} that early deposition of a significant amount of fibrin in superficially eroded plaques involves the TF-dependent extrinsic pathway of coagulation. This process may be better explained if one considers the role of TF-bearing apoptotic endothelial cells and microparticles in the pathophysiology of plaque erosion and thrombosis. This interesting hypothesis deserves to be tested in future experimental studies.

Despite this large body of evidence for a prothrombogenic role of apoptosis in vascular disease, recent experimental models in which massive apoptosis was induced within the vessel did not mention the occurrence of thrombosis.\textsuperscript{9,44} This seems at odds with our hypothesis. However, it is noteworthy that apoptotic death was specifically induced in neointimal smooth muscle cells of nonruptured plaques through seeding of vascular smooth muscle cells overexpressing the Fas-associated death domain or through downregulation of mitochondrial bcl-x\textsubscript{L} expression with the use of antisense oligonucleotides.\textsuperscript{9,44} Endothelial cells were not reported to be apoptotic in these models.\textsuperscript{9,44} Because there was no direct contact between apoptotic cells and the circulating blood, the absence of thrombosis is not surprising in these experimental conditions.

### Apoptosis as a Contributor to Blood Thrombogenicity

Besides the classic paradigm that coagulation is triggered after exposure of vessel-wall TF to the circulating blood after vessel damage, there is recent evidence that acute thrombosis may be initiated by TF originating from the circulating blood.\textsuperscript{,45} Giesen et al\textsuperscript{45} have elegantly shown a TF-dependent fibrin-rich thrombus formation on pig arterial media (which contains no stainable TF) and on collagen-coated glass slides (devoid of TF) after exposure of these surfaces to flowing native human blood. Active TF was isolated from freshly collected whole blood and was shown to originate mainly from circulating leukocytes. These cells are thought to be activated, at least at the time of thrombus formation, leading to deencryption of the cell-surface TF. Indeed, within the thrombus, TF was shown to be released in vesicular structures and can be transferred from leukocytes to platelet membranes through CD15 and TF-mediated interactions,\textsuperscript{46} potentially leading to the formation of TF-platelet hybrids, a phenomenon that would be critical to thrombus propagation.\textsuperscript{45}

In addition to activated leukocytes, we believe that apoptosis may also markedly contribute to the shedding of TF-bearing vesicles and hence to the thrombogenicity of the circulating blood. Apoptotic cells are the prototype of cells that can embolize into the circulation. Among the first morphological changes after initiation of the apoptotic process are membrane blebbing with shedding of microparticles, loss of focal adhesion sites, and retraction from the matrix followed by detachment.\textsuperscript{47} After plaque rupture and exposure of the plaque gruel, all vessel wall constituents, including TF-bearing apoptotic cells and microparticles, if present, can embolize into the circulation.\textsuperscript{2} Also, even in the absence of plaque rupture, luminal endothelial cells undergoing apoptosis,\textsuperscript{39} which may have already attracted activated platelets and fibrin, will finally detach and embolize into the circulating blood. Moreover, a proportion of viable endothelial cells that have detached from the extracellular matrix may become apoptotic,\textsuperscript{48,49} probably because of the loss of contact with antiapoptotic components of the vessel wall.\textsuperscript{47} If true, all these apoptotic cells and microparticles will eventually circulate in the peripheral blood and, in concert with activated cells and platelets,\textsuperscript{50,51} will participate in the dissemination of the procoagulant potential, contributing to blood thrombogenicity.

Several lines of evidence support the hypothesis that embolization of apoptotic or activated cells and microparticles may play a significant role in blood thrombogenicity. Recently, we examined the peripheral blood of coronary and noncoronary patients for the presence of circulating PS-bearing microparticles. Patients with ACS had a significant increase in circulating microparticles compared with stable coronary patients.\textsuperscript{52} Structurally, these microparticles resemble those extracted from human atherosclerotic plaques, but their tissue origin is presently unknown. A significant proportion of the microparticles was of endothelial or platelet origin. This may reflect the endothelial erosion at the site of
plaque disruption, the endothelial injury on exposure of plaque microvessels to inflammatory cells, or the injury associated with myocardial ischemia. Yet the importance of each of these potential factors is unknown. Patients with ACS have elevated levels of circulating TF,43 and there is evidence that acute thrombosis may be initiated by membrane-bound circulating TF originating from activated or injured cells.45 We believe that a major source of blood-borne TF could be the circulating microparticles that are endowed with potent procoagulant potential attributable to the presence of PS at their surface. A significant increase in the number of circulating endothelial cells, some of them being apoptotic,49 has also been reported in patients with ACS.50 These circulating cells and carcasses could represent those cells that have desquamated from the basal membrane in the early stages of apoptosis and that will engage into an apoptotic process once in the bloodstream. Taken together, these findings from different groups highlight the potential role of cell injury and apoptosis and the shedding of circulating microparticles in the pathophysiology of ACS. It is noteworthy that systemic disorders or conditions characterized by increased rate of apoptosis or increased circulating apoptotic microparticles54–57 (ie, disseminated lupus erythematosus, antiphospholipid syndrome, and cocaine abuse) are important risk factors for coronary thrombosis. The circulating procoagulant microparticles may also contribute to blood thrombogenicity factors for coronary thrombosis. The circulating procoagulant microparticles may also contribute to blood thrombogenicity of patients with hyperlipidemia or high blood glucose concentrations; these vascular risk factors are known to be responsible for increased apoptotic activity in vitro.58–60

In addition to their direct effect in promotion or amplification of the coagulation cascade, the circulating microparticles may also act in a variety of intercellular adhesion and activation processes and may participate in the long-range transmission of information to sites remote from the microenvironment of their formation.23,61–63 Circulating markers of inflammation are good predictors of vascular risk,64 and ACS are associated with a systemic inflammatory reaction. The circulating microparticles might contribute to such alterations.

Clinical Implications
Thrombus formation on a disrupted atherosclerotic plaque is the major threatening event in atherosclerotic disease. There is a growing body of evidence suggesting that apoptosis, through its procoagulant and proadhesive potentials, may play a critical role in both plaque and blood thrombogenicity and may be an important step in the transition from stable to unstable atherosclerotic disease. The recognition of the importance of apoptosis in atherothrombosis may lead to the development of new diagnostic and prognostic markers in acute ischemic syndrome evaluation. Circulating apoptotic and nonapoptotic microparticles could be a valuable marker of thrombus formation and hence of the instability of the atherosclerotic plaque. This hypothesis is now being tested in a multicenter study. On the other hand, diagnostic techniques aiming at the detection of apoptotic death in vivo67 may be of great value in the identification of unstable atherosclerotic plaques.

The recognition of the importance of apoptosis in atherothrombotic disease may also lead to the development of new antithrombotic strategies aiming at the reduction of apoptotic death.47 In fact, many of the available therapeutic agents that have been shown to reduce the incidence or recurrence of ACS may have been active, at least in part, through reduction in apoptosis. Atherogenic lipoproteins have potent proapoptotic properties.58 A reduction in atherogenic lipid accumulation within the plaque may greatly decrease the level of apoptotic death within the plaque, therefore limiting the formation of the thrombogenic lipid core. In support of this view, Kockx et al66 recently observed a marked reduction in apoptosis in rabbit atherosclerotic lesions after 6 months of cholesterol withdrawal. Such a reduction in apoptosis may be an important mechanism of plaque stabilization after hypolipidemic drug therapy. Recently, angiotensin-converting enzyme inhibitors have been shown to significantly reduce the occurrence of myocardial infarction and stroke in humans.67 We postulate that a reduction in endothelial apoptosis and thrombosis, through inhibition of the endothelial proapoptotic effects of angiotensin II,68 might be one of the potential mechanisms responsible for this beneficial effect. Similarly, the beneficial atheroprotective effects of estradiol may result, at least in part, from the preservation of endothelial integrity and inhibition of endothelial cell apoptosis.69 This may also be the case of vitamins with antioxidant properties that inhibit oxidized LDL–induced endothelial apoptosis in vitro.50 Another powerful antioxidant and antiapoptotic product for many cell types, including endothelial cells, is the product of the heme oxygenase (HO)-1 gene.70,71 Deficiency in HO-1 is associated with severe endothelial damage,72 whereas upregulation of HO-1 is associated with expression of various cytoprotective genes, such as bcl-xL, and prevents transplant arteriosclerosis.73 Moreover, endothelial integrity in this setting is associated with the expression of anti-inflammatory cytokines, including interleukin-10.74 Finally, it should be kept in mind that the occurrence of apoptosis within human atherosclerotic plaques is highly dependent on the inflammatory balance.6 Therefore, in vivo (local) delivery of products with anti-inflammatory activity, such as interleukin-10, might be a sound strategy to deactivate inflammatory cells and reduce apoptotic death and its prothrombogenic properties, leading to plaque stabilization.

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