Molecular Imaging of Macrophage Cell Death for the Assessment of Plaque Vulnerability

Eduard M. Laufer, Mark H.M. Winkens, Jagat Narula, Leonard Hofstra

Abstract—The ability to identify atherosclerotic plaques that are prone to rupture, also called vulnerable plaques, may provide a major step forward in the recognition of patients that have a high risk of developing acute myocardial infarction. Current clinical risk profiling algorithms, such as the Framingham and Procam risk scores, have reasonable predictive value in the assessment of the 10 year risk. These clinical risk profiling scores typically classify patients into low risk (10-year risk, less than 5%), intermediate risk (5% to 20% risk), and high risk (greater than 20%). The challenge to imagers is to identify the risk that is beyond 2% yearly risk. Molecular imaging may help identify plaque inflammation and apoptosis of inflammatory cells, which are obligatory components of the plaque instability. These processes offer specific biological targets that can potentially be exploited to obtain biological information on atherosclerosis development in the individual patient. (Arterioscler Thromb Vasc Biol. 2009;29:1031-1038.)

Key Words: atherosclerosis ■ molecular imaging ■ plaque biology ■ apoptosis

Atherosclerotic disease remains killer number one in both developed and developing nations. It afflicts women and men alike. Because of the epidemic proportions of obesity and aging of the population, it is expected that the impact of atherosclerotic disease will remain high in the new century as well. The first signs of the impact of the escalating proportions of obesity may have influenced recent event rates in the United States, wherein the decline in the incidence of acute myocardial infarction in men has come to a halt, and in women the numbers have started to escalate.

The main challenge for clinicians is to identify individuals who have a high likelihood of developing myocardial infarction. Clinical risk profiling algorithms, such as the Framingham and the Procam risk scores, have proven to be useful tools for the identification of individuals with a high risk to develop acute myocardial infarction in the next 10 years. Typically, the clinical risk scoring profiles categorize individuals in low risk (less than 5% 10-year risk), intermediate risk (5% to 20%), and high risk (20% or more) to develop acute myocardial infarction.1 However, clinical risk profiling does not provide a clear answer about the time frame when events may occur in the next 10 years. We may also need to find ways to improve the area under the ROC curve to enhance predictive value. Recent studies have shown that the addition of novel serum biomarkers to clinical risk profiling had limited beneficial effect, and that novel strategies need to be developed to improve the predictive value of diagnostic algorithms.2

One way to obtain better predictive values and more precise information is the use of imaging technology for identification of plaques that are vulnerable to rupture.3 Traditional imaging methods have provided excellent information about the location and the extent of luminal narrowing of atherosclerotic lesions.4 Clinical research studies have demonstrated that the occurrence of acute myocardial infarction is related to plaque characteristics. In at least 70% to 80% of cases, acute myocardial infarction is attributable to rupture of a vulnerable plaque, resulting in local thrombus formation and acute disruption in the coronary blood supply in the affected area.5 These studies have shown that vulnerable plaques are characterized by a large necrotic core, a thin fibrous cap, and by the presence of macrophages.6 Cell death of inflammatory cells by the process of apoptosis has recently been proposed to contribute to plaque vulnerability. Apoptosis has been linked to thinning of the fibrous cap, the development of the necrotic core, and positive vessel remodeling.7 Identification of apoptosis in atherosclerotic lesions may help identify vulnerable atherosclerotic plaques in patients. Because apoptosis is characterized most prominently by the phosphatidylserine (PS) shift from the intracellular leaflet to the extracellular leaflet of the plasma cell membrane,8 externalized PS provides an attractive target for apoptosis detection. Annexin A5, a normally circulating protein, demonstrates a strong affinity for PS in the nanomolar range and has proven to be an effective means to detect apoptosis both in vitro as well as in vivo.9

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Apoptosis in Plaque Vulnerability

A number of key characteristics in plaque vulnerability are related to apoptotic cell death. A large part of the necrotic core is likely derived from cell death of macrophage foam cells, and the cell death of smooth muscle cells leads to thinning of the fibrous cap and positive remodeling of the involved coronary segment. The thin-capped fibroatheroma has been defined as an atherosclerotic lesion with a fibrous cap of 65 μm or less. The fibrous cap is made up of collagen, which is mainly derived from smooth muscle cells (SMCs) of the plaque. The extracellular collagen helps to develop a stable and thick fibrous cap, which prevents exposure of the thrombogenic necrotic core to the blood. Regular loss of SMCs through apoptosis perpetuates attenuation and weakening of the fibrous cap. Further, it has been demonstrated that extensive and collective apoptosis of macrophages at the site of plaque rupture is associated with the acute coronary event. Based on these observations it appears that apoptosis and inflammation are substrates for plaque disruption.

In the study of culprit lesions obtained from victims of sudden coronary death, the plaques were divided into 2 mechanisms of death: ruptured plaques with acute thrombosis and stable plaques with and without healed myocardial infarction. Apoptotic cells were identified by staining of fragmented DNA, and expression and activation of 2 inducers of apoptosis, caspases-1 and -3. Unlike stable lesions, ruptured plaques showed extensive macrophage infiltration of the fibrous cap, particularly at rupture sites (Figure 1). Plaque rupture sites demonstrated a strong immunoreactivity to caspase-1 within the apoptotic macrophages; weak staining for caspase-3 was also observed. Although this study demonstrated extensive apoptosis of macrophages limited to the site of plaque rupture, it was not clear whether macrophage...
apoptosis is essential to acute plaque rupture or is a response to the rupture itself. Macrophage apoptosis may also facilitate the acute thrombotic event arising from the rupture itself. They captured the shed membrane microparticles originating from culprit carotid plaques as the products of apoptotic cells by using annexin A5. These microparticles were primarily monocytic in origin and very rich in tissue factor and hence procoagulant activity. Thus, it is conceivable that microparticles from macrophage apoptosis at the rupture site would induce thrombogenicity.

Feasibility of Imaging Apoptosis in Atherosclerotic Lesions
The proof of concept of exogenous radiolabeled Annexin A5 to image apoptosis and inflammation was provided in an experimental model of atherosclerosis in rabbits. Atherosclerotic lesions were formed by balloon deendothelialization of the infradiaphragmatic aorta, followed by 16 weeks of high-cholesterol high-fat diet. Control rabbits were studied without manipulation. In vivo imaging was performed after intravenous administration of 0.5 to 1 mg of Annexin A5 labeled with technetium-99m, followed by harvesting of the abdominal aortas, ex vivo imaging, and macro-autoradiography. In addition, histological examination was performed to localize the radiotracer in the atherosclerotic lesions. Two hours after injection of the radiolabeled tracer, there was clear demarcation of the radiotracer in the aorta by in vivo gamma imaging (Figure 2). Ex vivo imaging demonstrated a clear uptake of radiotracer in the aorta corresponding to the in vivo images and confirming the macroscopic localization of atherosclerotic lesions.
The accumulation of $^{99m}$Tc-Annexin A5 in atherosclerotic lesions in the region of the aorta affected by vascular injury was about 9.3-fold greater than in aortas of the control animals. The mean (±SEM) percent-injected dose per gram uptake in the aortas with atherosclerotic lesions (0.054± 0.0095%) was significantly higher than the activity control specimens (0.0058±0.001, P<0.0001). The Annexin A5 uptake was significantly higher in advanced lesions (AHA type IV lesions), where the incidence of apoptosis was maximal. Furthermore, the uptake correlated with the extent of macrophage presence in the plaques. Although the rabbit model of atherosclerosis does not develop vulnerable lesions as seen in humans, the data indicate that Annexin A5 targets to macrophages in the plaque and is related to plaque apoptosis.

Because human vulnerable atherosclerotic lesions exhibit a great quantity of activated or apoptotic macrophages, these data suggest that Annexin A5 may be an imaging marker for plaque vulnerability in patients with atherosclerotic disease.

**Confirmation of Annexin A5 Uptake in Other Animal Models**

The imaging of apoptotic cell death has been demonstrated in various other animal models of atherosclerosis, including transgenic mice such as apolipoprotein E–deficient (apoE$^{-/-}$) and low-density-lipoprotein receptor–deficient (LDLR$^{-/-}$) mice, which develop spontaneous atherosclerotic lesions, and the swine model of coronary atherosclerosis.
produced by endothelial injury and high-cholesterol diet. Radiolabeled Annexin A5 was successfully used for noninvasively image imaging in transgenic apoE−/− and LDLR−/− mice with or without high-cholesterol diet.14 The Annexin A5 uptake was compared with normal wild-type (C57BL/6) mice of the same genetic background. Noninvasive imaging was performed using micro–SPECT-CT, followed by ex vivo imaging of the explanted aorta and quantitative assessment of the radiotracer localization by gamma counting of the percentage injected dose per gram (%ID/g) annexin uptake (Figure 3). Subsequently, histological and immunohistochemical characterization of the aortic samples was performed. Aortic lesions were clearly visualized noninvasively by micro-SPECT, and aorta calcification was detected by micro-CT. The quantitative uptake of Annexin A5 was highest in the cholesterol-fed apoE−/− mice, followed by the normal chow-fed apoE−/−, the cholesterol-fed LDLR−/−, and the disease control normal mice. The histological extent of atherosclerosis paralleled radiotracer uptake, and immunohistochemical studies revealed a significant correlation between radiotracer uptake and both macrophage infiltration and the extent of apoptosis. Intravenously injected biotinylated Annexin A5 localized in apoptotic and nonapoptotic macrophages.

One of the major challenges is to develop a noninvasive imaging technology that is able to detect vulnerable plaques in the coronary tree. In a study in a swine model of atherosclerosis it was shown that the visualization of Annexin A5 uptake in the coronary tree is feasible using nuclear imaging.15 In male swine, atherosclerosis was produced by high-fat diet combined with vascular injury to the coronary vessels. After an average of 2 months, the swine underwent coronary angiography, followed by radiolabeled Annexin A5 and SPECT imaging. After in vivo imaging the animals were euthanized, the coronary vessels imaged by autoradiography and counted in the well counter. Immunostaining was performed for α-actin and caspase 3. Histopathology showed that the coronary lesions were early, mild, and mainly characterized by smooth muscle cells. Out of the abnormal vessels, 60% showed focal uptake of Annexin A5 in vivo, which corresponded to uptake on autoradiography. The count ratio of the injured vessel compared to control vessel was almost 3:1 for positive scans and 1.3:1 for negative scans. Scans positive for Annexin A5 uptake correlated with caspase-positive staining. These data provide proof of concept that noninvasive detection of the Annexin A5 uptake in atherosclerotic lesions of the coronary tree is feasible.

Clinical Feasibility of Apoptosis Imaging
In a first attempt to evaluate apoptosis imaging in a clinical setting, Annexin A5 imaging was performed in patients with recent (n=2) or remote history (n=2) of transient ischemic attack (TIA), respectively, 1 to 3 days before the scheduled carotid endarterectomy.16 Six hours after injection of 600 to 800 MBq 99mTc-Annexin A5, SPECT imaging was performed. The 2 patients who had suffered from a TIA 3 to 4 days before imaging showed clear Annexin A5 uptake (Figure 4) in the area of the culprit carotid artery lesion, as was identified by ultrasound. Histopathologic assessment of the endarterectomy tissue showed vulnerable plaque morphology, including substantial macrophage infiltration and intraplaque hemorrhage. Immunohistochemical evaluation showed binding of Annexin A5 predominantly to macrophages. The 2 patients, who had a remote history of TIA 3 to 4 months before imaging, showed no Annexin A5 uptake in the carotid artery on SPECT imaging. These patients had a similar carotid lesion as compared to the patients with a recent history of TIA. These patients had been treated with
statins and antiplatelet agents after the acute event. Endarterectomy specimens from the patients with a remote history demonstrated stable plaque characteristics with nonsubstantial macrophage infiltration and no intraplaque hemorrhage; only slight Annexin binding was observed on immunohistochemical analysis. These pilot data demonstrate that 99mTc-Annexin A5 targets to macrophage-rich lesions of plaques with vulnerable plaque morphology. In addition, the data show that noninvasive Annexin A5 imaging of vulnerable atherosclerotic lesions is clinically feasible and may recognize patients at risk for acute vascular events.

Acute and Chronic Resolution of Apoptosis in Atherosclerotic Plaques

Although apoptosis within atherosclerotic plaques is associated with plaque vulnerability and rupture, it has been proposed that the role of inhibition of the apoptotic process can be clarified by evaluating the impact of interventions which result in favorable outcomes. As such the influence of dietary modification and statin therapy on the incidence of apoptosis in experimental atherosclerotic lesions was seen. A group of rabbits with experimental atherosclerotic lesions was randomized as follows: high-cholesterol diet for 4 months (untreated atherosclerotic group), high-cholesterol diet for 3 months and normal chow diet for 1 month (diet withdrawal group), and high-cholesterol diet for 4 months and simvastatin orally every day of the last month (statin therapy group). Radiolabeled Annexin A5 was used for the noninvasive detection of the extent of apoptosis. For control experiments, either radiolabeled mutant Annexin A5 was used in the hypercholesterolemic rabbits or Annexin A5 in normal rabbits receiving normal chow. Quantitative Annexin A5 uptake in the abdominal aorta was determined and compared with the histological and immunohistochemical characteristics of the atherosclerotic lesions. Stabilization of atherosclerotic lesions in diet withdrawal and statin therapy groups is evident by increased SMC content, decreased macrophage populations, and decreased apoptosis after diet withdrawal (middle row) and statin therapy (bottom row). The statin group is significantly superior to the DW group. (Magnification ×100 for top row and ×35 for the middle and bottom). Modified with permission from Hartung et al.19

Figure 5. Annexin A5 uptake within abdominal aortas in various study groups. Ex vivo images of explanted aortas showed no Annexin A5 uptake in normal aorta (N-Annexin A5), but intense Annexin A5 uptake within abdominal aortas in rabbits fed high-cholesterol (HC-Annexin A5) diet for 4 months. There was only a negligible Annexin A5 uptake seen in diet withdrawal (DW-Annexin A5) and statin (Rx-Annexin A5) therapy group. Similar to the control group, no Annexin A5 uptake was observed in rabbits receiving high-cholesterol diet for 4 months and imaged with mutant Annexin A5 (HC+mAnnexin A5). Bar graphs (below) show quantitative Annexin A5 uptake within abdominal aortas in various study groups, represented as mean±SD %ID/g. Annexin A5 uptake corresponds to ex vivo images as above. Maximum Annexin A5 uptake (%ID/g) was seen in HC animals (P=0.0001) compared with N animals (controls). Uptake was significantly lower in DW and RX groups than in HC animals. No significant difference was observed between animals in DW and RX groups or between N and HC-mAnnexin A5 groups (P was not significant [NS]). Quantitative results confirmed ex vivo imaging observations. Photomicrographs in right panel of abdominal atherosclerotic lesions are stained for histology (with Movat pentachrome stain), macrophages (with Ram-11), alpha smooth muscle actin (with HHF35), and apoptosis (ISEL) from untreated atherosclerotic animal of HC-Annexin A5 (top), DW (middle), and Rx (bottom) groups. HC group demonstrates AHA type IV lesion with well-formed necrotic core in neointimal region. Staining with antibody specific for macrophages (CD68; RAM-11) shows widespread presence of macrophage infiltration in neointima. Staining with antibody against actin isotypes α and β (HHF-35) shows absence of SMC in neointima. SMC staining is visible only in normal medial layer of atherosclerotic vessel. DNA fragmentation staining (blue–black nuclei; arrow) by in situ end labeling (ISEL) and methyl green counterstaining demonstrate extensive apoptosis of macrophages. Stabilization of atherosclerotic lesions in diet withdrawal and statin therapy groups is evident by increased SMC content, decreased macrophage populations, and decreased apoptosis after diet withdrawal (middle row) and statin therapy (bottom row). The statin group is significantly superior to the DW group. (Magnification ×100 for top row and ×35 for the middle and bottom). Modified with permission from Hartung et al.19
A5 or mutant Annexin A5 was seen in rabbits on the normal chow diet or atherosclerotic rabbits, respectively (Figure 5). Because apoptosis of macrophages contributes significantly to plaque rupture, it is logical to presume that acute repression of apoptosis may be of therapeutic benefit in acute coronary events. Such a proposal may be of added value because resolution of apoptosis has also been shown to be beneficial in limiting myocardial injury in experimental myocardial infarction. Resolution of apoptosis by statins takes at least 4 weeks, a period wherein most recurrences of ischemic events are seen. As such, the role of caspase inhibitors on acute resolution of apoptosis in atherosclerotic lesions has been evaluated by imaging with Annexin A5. For this purpose, a large number of atherosclerotic rabbits received broad caspase, caspase-1, caspase-3, caspase-8, or caspase-9 inhibitors. These caspase-treated animals were compared with the magnitude of apoptosis in the untreated animal group and normal disease control animals. Radiolabeled Annexin A5 traced the atherosclerotic lesions best in untreated atherosclerotic rabbits. Quantitative Annexin A5 uptake, defined as the percent of injected dose per gram of abdominal aorta tissue, was significantly higher in untreated atherosclerotic animals compared with the normal rabbits or atherosclerotic rabbits receiving mutant Annexin. Among all caspase inhibitor-treated rabbits, uptake was 40% lower than the untreated atherosclerotic animals. In fact, the uptake was significantly lower in the rabbits receiving broad caspase or caspase-1, -3, or -9 inhibitors. Caspase-8 inhibitor did not affect apoptosis. On histological characterization, a substantial decrease in macrophage apoptosis was observed in caspase-inhibited animals. Because caspase-8 inhibition had no significant effect on apoptosis, and caspase-9 inhibition significantly blocked it, the apoptosis of foam cells is likely to involve the mitochondrial pathway and not the death receptor.

Figure 6. The extent of apoptosis in various animal groups represented by quantitative Annexin A5 uptake and histological characterization of untreated and broad caspase-inhibited animals. Modified with permission from Sarai et al.18 The left panel demonstrates the uptake of Annexin A5 in abdominal aortas. Disease control normal animals (N) with no atherosclerotic lesions are represented by an open bar, and the remaining atherosclerotic animals by solid bars. Atherosclerotic animals imaged with mutant Annexin were used as tracer controls (gray bar), and the remaining atherosclerotic animals were either untreated (black bar) or treated with various caspase inhibitors (colored bars). Broad and caspase-3 inhibition bars are shown in pink, caspase-8 and -9 inhibitors differentiating between mitochondrial and extramitochondrial pathways are in blue, and caspase-1–specific inhibition is represented by a green solid bar. The statistical significance of caspase inhibition, in comparison with the disease control and untreated atherosclerotic animals, is shown above and below the bars, respectively. The uptake is significantly higher in the abdominal aortas of untreated atherosclerotic animals compared with that in the disease and tracer control groups. Treatment with a broad caspase inhibitor or selective caspase-1, -9, or -3 inhibitor significantly reduced the apoptotic activity as represented by lower Annexin uptake. On the other hand, selective caspase-8 inhibition did not affect apoptosis. The right panel compares histological characterization of atherosclerotic lesions in atherosclerotic untreated animals and atherosclerotic animals receiving the broad caspase inhibitor. Movat pentachrome (M5Ch), smooth muscle cell (α-actin), and macrophage (Ram-11) staining (×200) in untreated atherosclerotic animals (left) and broad caspase inhibitor–treated animals (right) demonstrate similar cholesterol crystal-rich necrotic core, foam cell-rich, and smooth muscle-deficient lesions. The morphological characteristics of the lesions in untreated and caspase-treated atherosclerosis are unchanged. However, terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) reveals evidence of marked apoptotic nuclei in untreated atherosclerotic animals but marked resolution in the atherosclerotic lesions in caspase-inhibited animals.
pathway (Figure 6). In addition, the caspase-1 inhibitor also blocked apoptosis to a level similar to the broad caspase and specific caspase-3 inhibitors, suggesting that caspase-1 may also be involved in apoptosis in atherosclerotic lesions; this confirms the initial observations of caspase-1 activations in the victims of sudden death attributable to plaque rupture. Activation of caspase-1 in apoptosis in atherosclerosis in the absence of caspase-8 involvement suggests its interaction with mitochondrial pathway of apoptosis. Such interaction has recently been demonstrated in traumatic brain injuries, wherein increased levels of cytochrome c, caspase-3, and caspase-1 have been observed in the cerebrospinal fluid, and caspase-3 and -1 activation in brain tissue. It is likely that reactive oxygen species generation, which is downstream of caspase-1, plays an important role in caspase-1 based apoptotic cascade.

Conclusions
Molecular imaging strategies have been developed to detect apoptosis, an important pathological substrate of plaque instability. Some of these techniques have been used in clinical settings and demonstrate the possibility of their application in risk-stratification of asymptomatic subjects falling in the high-risk categories (more than 20% 10-year risk). In addition to the potential diagnostic application, molecular imaging also helps to better understand the pathogenesis of vulnerable plaques because it is the only strategy that can be used for the study of subcellular events in living organisms.

Disclosures
None.

References
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