Targeting of Apoptotic Macrophages and Experimental Atheroma With Radiolabeled Annexin V
A Technique With Potential for Noninvasive Imaging of Vulnerable Plaque

Frank D. Kolodgie, PhD; Artiom Petrov, PhD; Renu Virmani, MD; Navneet Narula, MD; Johan W. Verjans, MS; Deena K. Weber, BS; Dagmar Hartung, MD; Neil Steinmetz, MD; Jean Luc Vanderheyden, PhD; Mani A. Vannan, MD; Herman K. Gold, MD; Chris P.M. Reutelingsperger, PhD; Leo Hofstra, MD, PhD; Jagat Narula, MD, PhD

Background—Apoptosis is common in advanced human atheroma and contributes to plaque instability. Because annexin V has a high affinity for exposed phosphatidylserine on apoptotic cells, radiolabeled annexin V may be used for noninvasive detection of apoptosis in atherosclerotic lesions.

Methods and Results—Atherosclerotic plaques were produced in 5 rabbits by deendothelialization of the infradiaphragmatic aorta followed by 12 weeks of cholesterol diet; 5 controls were studied without manipulation. Animals were injected with human recombinant annexin V labeled with technetium-99m before imaging. Aortas were explanted for ex vivo imaging, macroautoradiography, and histological characterization of plaque. Radiolabeled annexin V cleared rapidly from the circulation (T1/2, 92 ± 25 minutes). There was intense uptake of radiolabel within lesions by 2 hours; no uptake was seen in controls. The results were confirmed in the ex vivo imaging of the explanted aorta. Quantitative annexin uptake was 9.3-fold higher in lesion versus nonlesion areas; the lesion-to-blood ratio was 3.0 ± 0.37. Annexin uptake paralleled lesion severity and macrophage burden; no correlation was observed with smooth muscle cells. DNA fragmentation staining of apoptotic nuclei was increased in advanced lesions with evolving necrotic cores, predominantly in macrophages; the uptake of radiolabel correlated with the apoptotic index.

Conclusions—Because annexin V clears rapidly from blood and targets apoptotic macrophage population, it should constitute an attractive imaging agent for the noninvasive detection of unstable atherosclerotic plaques. (Circulation. 2003;108:3134-3139.)

Key Words: apoptosis • atherosclerosis • imaging • nuclear medicine

There is emerging evidence that apoptosis contributes to the instability of the atherosclerotic lesion. Plaques vulnerable to rupture typically have a large necrotic core and an attenuated fibrous cap, which is significantly infiltrated by macrophages and lymphocytes; the smooth muscle cell (SMC) content within the fibrous cap is sparse.1 Although the consequences of cell death within the advance lesion are not precisely defined, morphological data suggest that apoptosis of macrophages contributes substantially to the size of the necrotic core,2 whereas apoptosis of SMCs results in thinning of the fibrous cap.3 It has been recently proposed that extensive apoptosis of macrophages occurs at sites of plaque rupture and possibly contributes to the process of rupture.4 Therefore, detection of apoptosis may help identify lesions prone to rupture.

Apoptosis is associated with molecular alterations in the cell membrane, condensation of cytoplasmic proteins, and DNA fragmentation. Cell membrane changes comprise loss of phospholipid asymmetry, resulting in the exposure of phosphatidylserine (PS) on the cell surface.5 Expression of PS allows clearance of the unwanted cells by the phagocytes. Annexin V, a naturally occurring protein, has a high affinity for binding to PS.6 Annexin V can be conveniently radiolabeled with technetium (99mTc) and has been used for the noninvasive detection of apoptosis in myocardial infarction and inflammatory myocardial diseases.7 Because apoptosis
may be a marker of plaque instability, we reasoned that the radiolabeled annexin V should allow the noninvasive detection of rupture-prone lesions. Because there is no available animal model of plaque vulnerability, we used $^{99m}$Tc-labeled annexin V for the imaging of experimental atherosclerotic lesions in the hypercholesterolemic rabbit as a proof of concept. Apoptosis, predominantly in the macrophage population, has been identified in rabbit plaques.

**Methods**

**Induction of Experimental Atherosclerosis in Rabbits**

Male New Zealand white rabbits weighing 2.5 to 3.0 kg were obtained from Charles River Breeding Laboratories (Wilmington, Mass). Animals were housed under the current guidelines of the NIH; the Drexel University animal research review committee approved the study protocol. After a 2-week quarantine, rabbits were fed a 1% cholesterol diet custom-mixed in 6% peanut oil; the noninjured control animals were fed normal rabbit chow. One week after initiation of the high-fat diet, rabbits underwent deendothelialization of the infradiaphragmatic aorta by modified Baumgartner technique. Animals were anesthetized with ketamine and xylazine (100 mg/mL, 10:1 vol/vol; 1.5 to 2.5 mL SC). The right femoral artery was surgically exposed, a small arteriotomy site created, and a 4F Fogarty embolectomy catheter (12-040-4F; Edwards Laboratories, Inc.) was advanced into the abdominal aorta up to the level of the diaphragm. The catheter was then inflated to a pressure of 3 psi with radiographic contrast medium and pulled antegrade to the bifurcation of the aorta. After 3 such passes, the surgical cutdown site was closed with a 3-0 biodegradable suture. After recovery from the anesthesia, the animals were returned to their cages and continued on the hyperlipidemic diet for 11 more weeks. Ten rabbits were used for imaging experiments. Atherosclerotic lesions were induced in 5 animals, and the remaining animals were studied without manipulation.

**Radiolabeling of Annexin V With $^{99m}$Technetium**

Human recombinant annexin V was produced by expression in *Escherichia coli* and was radiolabeled with technetium-$^{99m}$Tc as described previously. Recombinant annexin V has been shown to retain PS-binding activity equivalent to that of native annexin V. Before radiolabeling, annexin V was derivatized with the nicotinic acid analog hydrazinonicotinamide (HYNIC, Anor Med) at a 0.9-mol/mol ratio by gently mixing. HYNIC is a bifunctional molecule with an affinity for lysine residues of proteins on 1 moiety and to the conjugates of $^{99m}$Tc on the other; the stable complex formed by this molecule did not affect protein bioreactivity. To bind $^{99m}$Tc to the HYNIC-annexin V conjugate, a reduced tin (stannous ion) and tricine solution was added to $^{99m}$Tc pertechnetate with an aliquot of HYNIC-annexin V under anoxic conditions. The final specific radioactivity was 10 to 200 μCi/μg protein (1 μCi=37 kBq). Thin-layer chromatography using the solvent NaCl showed a radio-purity of 92% to 97%. For the present study, annexin V kits were obtained from Theseus Imaging, Inc.

**Molecular Imaging of Experimental Atherosclerosis With $^{99m}$Tc-Labeled Annexin V**

For molecular imaging, 1 mg of annexin V labeled with 12±4 μCi of $^{99m}$Tc was administered intravenously. Serial blood samples were drawn for blood clearance. Planar whole-body images were captured using a gamma camera in the left lateral position at 5 and 30 minutes and 1, 2, and 3 hours. After obtaining the images, the rabbits were euthanized with overdose of sodium pentobarbital (120 mg/kg IV). The entire length of the aorta was exposed and cleaned of adherent fat and connective tissue before removal.

**Ex Vivo Assessment of Annexin V Uptake in the Atherosclerotic Lesions**

The aorta was removed, opened longitudinally along the ventral surface, and imaged ex vivo by laying flat on the gamma camera. The ROI analysis of the ex vivo images was performed for the comparison of annexin uptake in lesion and normal aortic regions. Gross pathologic lesions were traced on clear acetate sheets for comparison with the imaging or autoradiographic results. The aorta was then divided into 5 regions, ascending, arch, descending thoracic, superior abdominal aorta, and inferior abdominal aorta. These segments were weighed and counted in an automatic well-type gamma counter (model 1282 Compugamma; LKB Instruments, Inc) for determination of the percent injected dose of annexin per gram (%ID/g) of tissue. The aorta from 4 rabbits were then reconstructed and covered with a single layer of saran wrap and placed on high-speed x-ray film (Kodak Ortho-film OH-1) and stored at −70°C for 1 week. The radiographs were then developed in a 90-second X-OMAT processor.

**Histological Assessment of Experimental Atherosclerosis**

Frozen aortic segments were thawed and fixed with HEPES-buffered formalin (4%) with 2 mmol/L Ca$^{2+}$ added. From 4 rabbits, 20 lesions were histologically and immunohistochemically analyzed. Each specimen was subdivided into 3 equidistant sections and embedded on edge in paraffin. The tissue was then dehydrated in a graded series of ethanol. Serial 4-μm-thick sections were cut and mounted on charged slides (Fisher, Superfrost). Tissue sections were stained with H&E and Movat Pentachrome elastin stain. In addition, 2 specimens were also prepared for ultrastructural characterization of apoptosis.

**Definitions of Atherosclerotic Lesions**

Atherosclerotic lesions were characterized using a classification scheme based on the recommendations of the American Heart Association (AHA) and the American College of Cardiology (ACC). AHA type II lesions, also referred to as fatty streaks or intimal xanthomas, were plaques consisting of macrophage-derived intimal foam cells with SMC and extracellular matrix. AHA type III lesions, or pathologic intimal thickening, were lesions with focal acellular areas containing extracellular lipid pools in between SMCs and proteoglycans. AHA type IV lesions, also called fibrous cap atheromas, were plaques containing a necrotic core with prominent cholesterol clefts and overlying fibrous cap; occasionally the core region showed calcification within the deep intimal layers. Lesions in the rabbit model of balloon injury and hypercholesterolemia typically do not show hemorrhage, plaque rupture, or thrombosis.

**Immunohistochemical Staining for Cell Composition**

Sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide for 20 minutes to inactivated endogenous peroxidases. Tissue sections were then incubated in protein-free block (Dako) for 10 minutes to inhibit the nonspecific binding of primary antibody. Smooth muscle cells were identified using a primary antibody against actin isotypes α and β (HHF-35 Enzo, dilution 1:40, 1-hour incubation). Macrophages were localized using the marker RAM-11 (DAKO, dilution 1:200 overnight incubation). Primary antibodies were labeled with a biotinylated link antibody directed against mouse using a peroxidase-based kit (LSAB, DAKO). Immunohistochemistry was visualized by an AEC substrate-chromogen system (Dako) and counterstained with Gill’s hematoxylin. The specificity of primary antibody was confirmed both by omitting the primary antibody and by substituting isotype-matched antibodies.

Quantification of immunohistochemistry for smooth muscle cell actin and macrophages was performed by computer-assisted color image analysis (BIOQUANT, R&M Biometrics, Inc). A color threshold mask for the positive was established; positive areas are expressed as a percentage of total plaque area.
In Situ End-Labeling of DNA Fragmentation
In situ labeling of DNA fragmentation was performed using terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling based on an in situ apoptosis detection kit (TACS, Trevigen). Deparaffinized sections were treated with 0.3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase. The sections were rinsed and then digested with 20-μg of proteinase K (EM Science) for 12 minutes at room temperature. Exposed DNA fragments were labeled with biotinylated nucleotides (dNTPs) and TdT for 1 hour at 37°C. The incorporation of biotinylated nucleotides into DNA was detected with a streptavidin-conjugated horseradish peroxidase. A positive reaction was visualized with the chromogenic substance diaminobenzidine tinted with CoCl₂, producing a black reaction product. The sections were counterstained with methylgreen (blue-green nuclei).

For each arterial section, at least 300 total cells were counted in 4 random high-power fields, corresponding to a total area ranging from 0.13 to 0.20 mm². The numbers of apoptotic nuclei were expressed as a percentage of total cells (apoptotic index), and the mean values are reported according to the various lesion types.

Statistical Analysis
The values represent the mean±SEM. Comparisons among varying lesion types were performed using a 1-way ANOVA with post hoc analysis by Scheffé test. P<0.05 was considered significant.

Results
Noninvasive Detection of Experimental Atherosclerotic Lesions: In Vivo Imaging
The left lateral decubitus images of rabbits with experimental atherosclerosis injected with ⁹⁹mTc-labeled annexin V showed primarily blood pool activity shortly after administration (Figure 1A). At this time, lesion activity could not be distinguished from blood pool activity. At 2 hours, however, there was clear delineation of radiolabel within the abdominal aorta by in vivo gamma imaging (Figure 1B).

Ex Vivo Imaging
After explantation of the aorta, ex vivo imaging showed a robust uptake of radiotracer in the infradiaphragmatic aorta corresponding to the in vivo images and conforming to the macroscopic distribution of atherosclerotic lesions (Figure 1C). The uptake of radiolabel was absent in areas without grossly visible atherosclerotic lesions; these areas were predominantly localized to the nondeneded descending thoracic aorta. Macroautoradiography confirmed the ex vivo images and correlated with the atherosclerotic regions of the aorta in the explanted specimen.

In Vivo and Ex Vivo Imaging of Control Animals
The left lateral decubitus images of unmanipulated control animals showed blood pool activity similar to those animals with balloon injury and a high-fat diet (Figure 1D). In contrast to vessels with plaques, at 2 hours after administration, there was no localization of radiotracer within the presumably normal vessel wall (Figure 1E). Ex vivo imaging confirmed the lack of radiotracer uptake (Figure 1F) and correlated with the in vivo images, macroautoradiography, and lack of atherosclerotic lesions in the explanted aorta.

Quantitative Uptake of Annexin V in Lesions
The temporal blood clearance of radiotracer from the circulation was biexponential, with an initial fast component \( T_{1/2} \alpha \) of only 9 minutes followed by a slower component \( T_{1/2} \beta \) of 46 minutes. The accumulation of ⁹⁹mTc-labeled annexin V in atherosclerotic lesions in the balloon-denuded (abdominal) region of the aorta was 9.3-fold greater than in the corresponding control abdominal aortic region. The mean±SEM %ID/g uptake in the specimens with lesions (0.054±0.0095%) was significantly higher than the background activity in the normal specimens (0.0058±0.001, \( P=0.000 \)). The ROI analysis of the ex vivo images of aorta and comparison of the regions with atherosclerotic lesions and normal aortic segment demonstrated a 15±7-fold higher uptake in the lesion area. The lesion-to-blood ratio was 3.0±0.37, and normal aorta-to-blood ratio was 0.33±0.08. The biodistribution of radiotracer in nontarget organs showed maximum radiation burden in the renal cortex (6.4±0.9), followed by the spleen (0.41±0.09) and liver (0.16±0.04).

Histological Characterization of Atherosclerotic Lesions in Rabbits
Aortic sections from hypercholesterolemic animals demonstrated various atherosclerotic lesion types; approximately
20% of plaques were classified as AHA type II lesions, 30% as AHA type III lesions, and 50% as type IV lesions (Figures 2A through 2C).

Total SMC and macrophage burden in the various lesion types was quantified. The SMC burden among the AHA type II to IV lesions was comparable (0.54 ± 0.24, 0.64 ± 0.09, and 0.56 ± 0.14 mm², respectively). In contrast, total macrophage burden was increased approximately 2-fold in AHA type IV lesion (3.2 ± 0.93 mm²) compared with type II (1.6 ± 1.2 mm²) and type III (1.3 ± 0.73 mm²) lesions.

Correlation of Histological Characteristics and Annexin V Uptake

The uptake of ⁹⁹mTc-labeled annexin V was dependent on lesion severity (Figure 2D). The mean %ID/g uptake was significantly higher in aortic segments with AHA type IV lesions (0.034 ± 0.006) than type II (0.013 ± 0.002; P = 0.02) or type III lesions (0.0169 ± 0.0032; P = 0.03). Differences in radiotracer uptake between AHA type II and III lesions were not significant.

Regression analyses of a combined sample of aortic sections from all lesion types demonstrated no association between SMC content and radiotracer uptake (r = 0.08, P = 0.73) (Figure 2E). In contrast, there was a positive correlation between overall macrophage burden and uptake of radiolabeled tracer (r = 0.47, P = 0.004) (Figure 2F).

Identification of Apoptosis by DNA Fragmentation Staining

Apoptotic cells in AHA type II and III lesions were confined to the superficial layers of the plaque, whereas in type IV lesions, apoptosis was also prevalent in the deeper intimal layers of developing necrotic core (Figures 3A through 3D). Immunohistochemistry confirmed these regions to be rich in macrophage-derived foam cell; SMC content was negative. In contrast, rare apoptotic cells were found in the medial layer of all vessels examined. The mean apoptotic index for AHA type II lesions was 15.2 ± 3.2; for type III, 21.7 ± 2.8; and for type IV, 49.2 ± 5.8 (Figure 3E). Regression analysis showed a significant correlation between apoptotic index and radiotracer uptake (r = 0.56, P = 0.01) (Figure 3F).
Role of Apoptosis in Promoting Plaque Instability

Although the mechanisms underlying plaque instability are not well understood, recent literature supports the belief that cell death in plaques may promote rupture. Apoptosis occurs commonly in nonulcerated fibrous or fibrofatty lesions and is localized to inflammatory cells in the fibrous cap, deep intima, and shoulder regions; a fair degree of apoptosis is also found in macrophages surrounding the lipid core. It has been proposed that macrophage cell death may promote plaque instability by contributing to the size of the necrotic core. On the other hand, apoptosis of SMCs within the fibrous cap may represent a chronic process leading to fibrous cap thinning. Alternatively, SMC death may incite plaque instability by upregulation of monocyte chemotactic protein-1 and intense infiltration of macrophages in the fibrous cap.

In addition to contribution to plaque vulnerability, intimal cell death may actually precipitate acute coronary events. Analysis of coronary arteries from sudden coronary death patients in whom plaque rupture was the culprit lesion demonstrated extensive macrophage apoptosis localized to the site of plaque rupture. In contrast, apoptosis was minimal in the remote sites. Biochemical analysis showed selective activation of caspase-1, a promoter of apoptosis, in ruptured arteries compared with stable plaques. On the other hand, clinical studies have demonstrated increased circulating apoptotic cell death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol. 2000;20:1262–1275.

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Noninvasive Imaging of Apoptosis in Atherosclerotic Plaques

In earlier studies by light- and electron-microscopic autoradiography in hypercholesterolemic rabbits, the binding of 125I-labeled annexin V was increased in areas of atherosclerotic plaque and was predominantly localized to macrophages. These results are in agreement with our observations in ApoE mice, wherein we injected biotinylated annexin V that localized in apoptotic macrophages (MAC-3-positive and TUNEL-positive) in atherosclerotic lesions (data not shown). The unique binding affinity of annexin V for macrophages makes it a promising candidate for the noninvasive imaging of apoptosis within the atherosclerotic plaque.

In the present study, radiotracer uptake occurred preferentially in AHA type IV lesions, which demonstrated higher macrophage burden and increased prevalence of apoptosis compared with less advanced lesions. If apoptosis indeed contributes to plaque instability, then such a presumptive threshold for radiotracer uptake should be of significant clinical importance. Although current imaging techniques such as MRI or intravascular ultrasound provide better information on the morphological characteristics of the plaques, the molecular nuclear imaging may allow targeting of pathophysiological processes within the plaque, such as those critical to lesion instability.

Conclusions

The present study demonstrates the feasibility of targeting of apoptosis in the experimental atherosclerotic lesions in vivo. Because apoptosis is a potential determinant of plaque instability, annexin V imaging should constitute a useful noninvasive diagnostic tool, particularly if manipulation of apoptosis evolves as a strategy for plaque stabilization.

Acknowledgments

This study was supported by National Institutes of Health grant 1 ROI HL68657–01 (to Dr Narula).

References


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_Circulation_. 2003;108:3134-3139; originally published online December 15, 2003; doi: 10.1161/01.CIR.0000105761.00573.50

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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