In Vivo Imaging of Proteolytic Activity in Atherosclerosis

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**Background**—Atherosclerotic plaque rupture, the most important cause of acute cardiovascular incidents, has been strongly associated with vascular inflammation. On the basis of the hypothesis that the inflammatory response and proteolysis lead to plaque rupture, we have examined the role of cathepsin B as a model proteolytic enzyme.

**Methods and Results**—Using western-type diet–fed apoE and apoE/endothelial NO synthase double knockout mice as models of atherosclerosis, we show (1) that cathepsin B is upregulated in atherosclerotic lesions characterized by high degrees of inflammation compared with normal aorta or silent lesions, (2) that intravenously injectable novel cathepsin B imaging beacons are highly activated within active atherosclerotic lesions and colocalize with cathepsin B immunoreactivity, and (3) that cathepsin B activity in atherosclerotic lesions can be imaged in whole animals by using a novel near-infrared tomographic imaging system.

**Conclusions**—These studies indicate that cathepsin B, and potentially other proteases, may serve as a biomarker for vulnerable plaques when probed with beacons. The tomographic in vivo imaging method as well as catheter-based optical sensing methods could be readily adapted to screening and potentially to the molecular profiling of a number of proteases in vulnerable plaque in vivo. *(Circulation. 2002;105:2766-2771.)*

**Key Words:** atherosclerosis ■ enzymes ■ cathepsin B ■ imaging

Atherosclerosis remains a major health problem in the United States, with significant morbidity and an estimated annual mortality of 500,000 deaths from coronary artery disease alone.¹ The lesions in atherosclerosis represent a series of specific cellular and molecular responses best described as an inflammatory disease.²,³ The earliest lesion (fatty streak) is a pure inflammatory lesion consisting of monocyte-derived macrophages and T lymphocytes.⁴ The ubiquitous monocyte is present in every phase of atherogenesis, and hydrolytic enzymes secreted by these and other cells may play a central role in different stages of atherogenesis, in particular, the resorption of the fibrous cap leading to subsequent plaque rupture. Given the marked biological heterogeneity of plaque activity from one lesion to another, there has been a widespread interest in defining the characteristics and molecular basis underlying plaque rupture and thrombosis.⁵,⁶,⁷

Conventional imaging techniques largely assess luminal narrowing, calcification content, or morphological abnormalities of affected atherosclerotic lesions.⁷,⁸ It is now clear that luminal stenosis is a poor indicator of lesion activity or of lesions clinically at risk. Acute plaque rupture and subsequent thrombosis may occur in vulnerable plaques that do not appear anatomically severe, whereas other lesions that are more flow-limiting may be quiescent and do not progress. A fundamentally different approach of molecular imaging has been made possible through the development of enzyme sensing near-infrared imaging probes,⁹ which are quenched in their native state and become brightly fluorescent on enzyme cleavage.¹⁰–¹² Moreover, the detection of such probes in vivo has recently become possible by a novel imaging technique, fluorescence-mediated tomography (FMT).¹³ FMT is capable of sensing picomole to femtomole quantities of fluorochromes in deep tissues at macroscopic scale, ie, in whole animals with millimeter resolution. The technique shares tomographic principles with diffuse optical tomography¹⁴,¹⁵ but simultaneously uses absorption and fluorescence measurements for accurate 3D reconstruction of fluorochrome concentration.¹³

Atherosclerotic plaque rupture associated with inflammation has been correlated with the presence of highly activated macrophages.¹⁶ These cells are known to contain and secrete proteolytic enzymes that mediate vulnerable plaque erosion and rupture.¹⁷,¹⁸ Cathepsin B is a protease that is highly expressed in biologically active macrophages and, thus, in a number of inflammatory, dysplastic, and neoplastic disorders.¹⁹ We hypothesized that imaging of cathepsin B activity in atherosclerosis may serve as a new measure of plaque inflammation and vulnerability. To test this hypothesis, we used apoE knockout mice and apoE/endothelial NO synthase (eNOS) double knockout mice fed a western-type diet as

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animal models. Using an enzyme-sensitive activatable cathepsin B probe, we show remarkable correlation between probe activation, in vivo imaging, and histological evidence of cathepsin B protease activity in active plaques.

**Methods**

**Animals and Diet**

We used 3 different mouse models: apoE-deficient (apoE−/−) mice on a western-type diet (n = 10), apoE/eNOS double mutant mice (n = 5) on a western-type diet, and wild-type mice with a C57BL/6J genetic background on a regular diet (n = 5), which served as controls. The apoE−/− mice (Jackson Laboratories, Bar Harbor, Me) and eNOS−/− mice (Jackson Laboratories) were imaged for 20 generations to the C57BL/6J genetic background, and the offspring were genotyped for eNOS by Southern blotting and for apoE by the use of polymerase chain reaction (PCR). Both mutant mouse strains were fed a western-type diet for 24 weeks after they were weaned at 21 days. ApoE−/− mice developed typical atherosclerotic lesions resembling human active (vulnerable) plaques. ApoE/eNOS double mutant mice additionally developed abdominal aortic aneurysms and aortic dissection; the histological features of these mice have previously been described.

**Cathepsin B–Sensitive Imaging Beacon**

The cathepsin B–sensitive near-infrared fluorescence (NIRF) probe was based on a macromolecular assembly whose structure has previously been described. The probe was tested against human purified cathepsin B, against a panel of other cathepsins (H, L, and D), and in cell culture by using J774 macrophage-like cells (American Type Culture Collection).

**FMT Imaging**

To test whether cathepsin B probe activation could be imaged in intact animals, we imaged apoE−/− mice (n = 3) 24 hours after intravenous administration of the probe (5 nmol in 200 μL saline injected through the tail vein). The design and testing of the FMT imaging system and mathematical image reconstructions have been described in more detail elsewhere. In parallel, some animals were subjected to MRI to determine the location of the aortic arch and descending aorta (1.5 T GE Sigma home-built parallel wound solenoid radiofrequency mouse coils; fast spin echo, field of view 6 cm, 256×256 imaging matrix, slice thickness 1.5 mm, interleaved, echo time 17 ms, repetition time 500 ms, and 4 excitations).

**NIRF Reflectance Imaging and Lesion Assessment**

In addition to whole-body FMT imaging, all aortas (n = 20) were also imaged ex vivo by using a home-built NIRF reflectance imaging system. For these studies, the animals were first intravenously injected with the cathepsin B–imaging probe, and aortas were excised 24 hours later. In some animals, the aortas were imaged intact, whereas in other animals, they were opened longitudinally. To identify lipid-rich intraluminal lesions, the aortas were stained with Sudan IV.

**Histology, Immunohistochemistry, and Confocal Microscopy**

To evaluate the macroscopically observed atherosclerotic lesions, histology was performed on imaged aortas. Five-micrometer sections were stained with hematoxylin/eosin or Masson’s trichrome. Aneurysms were defined as an increase in vessel diameter of >50% and were evaluated according to the guidelines published by the Society for Cardiovascular Surgery. To evaluate local expression levels of cathepsin B in atherosclerotic lesions, immunohistochemistry was performed. A primary goat polyclonal anti-mouse IgG antibody was used (Santa Cruz Biotechnology) and revealed with a secondary antibody and diaminobenzidine (DAB, Vectastain).

To determine the cellular origins of cathepsin B production in histological sections, we also performed dual-label confocal microscopy. The sections were stained with an anti-macrophage antibody (rat anti-mouse Mac-3 monoclonal antibody–conjugated FITC, Pharmingen) and anti–cathepsin B (Santa Cruz Biotechnology). Specimens were visualized by confocal microscopy (Leica TCS NT4D) with the use of a BPS30/30 filter for FITC and a LP590 filter for Texas red.

**RNA Isolation and RT-PCR**

To determine the expression levels of cathepsin B in apoE−/− or wild-type aortas, reverse transcription (RT)-PCR was performed (n = 6 animals). Aortas were dissected, and samples were snap-frozen in liquid nitrogen. Total RNA was extracted by using Trizol (GIBCO-BRL) and reverse-transcribed by using Superscript IRT (GIBCO-BRL) and oligo(dT)15 priming. Cathepsin B and control β-actin reactions were always run on the same gel.

**Western Blot**

Western blotting of cathepsin B was performed by using a primary anti–cathepsin B polyclonal antibody (Bio-Rad Laboratories) and a secondary antibody conjugated with alkaline phosphatase (Sigma Chemical Co). Alkaline phosphatase activity was visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (NBT/BCIP, Boehringer-Mannheim).

**Results**

**Imaging Probe Is Activated by Cathepsin B**

The biocompatible NIRF imaging probe was selectively activated by cathepsin B compared with other cathepsins (including H, D, and L). The excitation and emission maxima were 673 nm and 689 nm, respectively, and the fluorescence signal was increased up to 50-fold after incubation with purified enzyme. The probe was not activated to any significant degree by either serum or whole blood within 24 hours of the incubations. The probe was clearly activated by macrophage-like J774 cells in culture. Interestingly, cell culture supernatant also activated the probe to a significant amount, indicating secretion of the enzyme into extracellular environments.

**Imaging of Atherosclerotic Lesions**

In the following experiments, the cathepsin B probe was injected intravenously into mice, and they were imaged 24 hours later. To determine whether the atherosclerosis-associated activation of the cathepsin B reporter probe could be detected in intact animals, we first performed FMT in a subgroup of apoE−/− animals. FMT has been recently developed as a quantitative 3D noninvasive imaging technique capable of sensing picomole to femtomole quantities of fluorochromes in deep tissues at macroscopic scale. As shown in Figure 1, significant fluorescence signal (submicrometer amounts) could be detected to be originating from within the aortic arch and the abdominal aorta of the apoE−/− mice after the mice had been injected with the beacon.

To correlate the signals observed in intact animals, aortas were excised and carefully analyzed. As shown in Figure 2a,
atherosclerotic lesions (white areas) were seen in the aorta, predominantly in the aortic arch, in the origins of the brachiocephalic, left subclavian, and left common carotid arteries, and throughout the abdominal aorta. NIRF imaging of the same vessels (Figure 2b) showed many of the atherosclerotic lesions to generate significant signal, consistent with activation of the probe by cathepsin B within the tissue. In contrast, control animals injected with saline did not show any significant fluorescence in atherosclerotic lesions. In all animals, there was heterogeneity of the NIRF signal correlated with cathepsin expression (see below). Animals not injected with the imaging probe and normal control animals showed no significant fluorescence in their aortas (see below).

We next quantified the intensity of the NIRF signal in aortic arch lesions, abdominal aortic lesions, and unaffected aortas in apoE−/− mice and apoE/eNOS−/− mice as well as in control mice. As shown in Figure 3, a significantly higher NIRF signal was noted in the arch lesions and abdominal aortic lesions compared with the unaffected aorta (P<0.001). There were no differences between the signal intensity of lesions in apoE−/− mice and apoE/eNOS−/− mice, and the animals were thus grouped. However, NIRF signal intensity was higher in the aortic aneurysms occurring only in apoE/eNOS−/− mice than in nonaneurysmal aortic atherosclerotic lesions (P<0.005).

Colocalization of NIRF Signal With Cathepsin B Immunohistochemistry
Cross sections of the aorta through atherosclerotic lesions showed cathepsin B immunoreactivity primarily within the intimal lesions in the areas adjacent to the vessel lumen.
(Figure 4a). The medial layer of the vessels was demarcated by the internal and external elastic laminae, and a prominent intimal layer was consistently identified (intimal hyperplasia). Cathepsin B immunoreactivity was highest (brown staining) in the areas next to the lumen (Figure 4c and 4d). Figure 3b shows NIRF images from adjacent sections of the same vessels showing colocalization of cathepsin B immunoreactivity. The elastin layers of the media showed fluorescence at 700 nm but also in other channels (eg, blue, green, and red channels) and thus could be easily distinguished electronically from more “monochromatic” NIRF originating from the near-infrared fluorochrome. Using confocal imaging and double-immunofluorescence labeling, we found that cathepsin B immunofluorescence also colocalized with immunoreactivity for Mac-3, a macrophage marker (Figure 5).

**Overexpression of Cathepsin B in Atherosclerotic Lesions**

To confirm that the NIRF signals reflect cathepsin B content, we also extracted RNA from unaffected aorta, nonaneurysmal aortic lesions, and aortic aneurysms. RT-PCR with the use of primers specific for cathepsin B confirmed that cathepsin B mRNA content was increased in aortic lesions over unaffected aorta and was highest in abdominal aortic lesions. Western blotting was also in accordance with the above results (Figure 6).

**Discussion**

The vascular biology underlying atherosclerosis and its acute complications, such as plaque rupture and thrombosis, is slowly emerging.1 One of the key definable events is leuko-
cyte recruitment, in particular, monocytes, presumably mediated by chemokines such as monocyte chemoattractant protein-1.23 Monocytes, once recruited to the arterial intima, take up lipids and become so-called foam cells. The remarkable population and replication of these cells in the intima are likely caused by factors such as macrophage colony–stimulating factor. As plaques progress, smooth muscle cells produce increased amounts of extracellular matrix, including collagens and proteoglycans, among others. Instead of progressive growth of intimal lesions leading to critical stenosis, it is now recognized that plaque erosion with fracture leading to thrombosis is a common cause of acute coronary syndromes. Plaque rupture is most likely due to an increased release of proteolytic enzymes, such as elastolytic cathepsins,17 matrix metalloproteinases,18 and other enzymes24,25 from macrophages.16

Results from the present study demonstrate inflammatory features similar to those described in other models of active atherosclerosis and, in addition, point to cathepsin B as a major source of proteolytic enzyme produced by intimal cells (Figures 3, 5, and 6). Although we have also observed overexpression of other proteolytic enzymes (eg, matrix metalloproteinase-2), cathepsin B expression was found to be a particular suitable biomarker because of its high local levels of expression. Similar observations have previously been observed for atherosclerosis17,26,27 and aortic aneurysm.28,29 Cathepsin B is a cysteine protease with lysine-lysine enzymatic activity (hence capable of activating fluorochromes from the poly-L-lysine backbone) and arginine-arginine enzymatic activity. It is present in many cell types and occurs in particularly high concentrations in macrophages. Within cells, the enzyme is located in high concentrations in lysosomes but also exists in the cytoplasm and on the cell surface and is also secreted in significant amounts.19 Because of its unique character, cathepsin B has been used as a biomarker to detect host response in tumors10 and to detect preneoplastic dysplastic lesions.30

Several invasive and noninvasive imaging techniques are available to assess atherosclerotic vessels. Most of the techniques identify the luminal diameter, volume, and thickness of the plaque and, occasionally, lipid and calcium contents. However, none of the current imaging techniques is capable of characterizing biological plaque activity to identify high-risk patients. Rupture-prone coronary plaques (vulnerable plaques) and high-risk carotid plaques tend to have a lipid-rich core and a thin cap, which have been assessed with catheter-based intravascular ultrasound, angioscopy, optical coherence tomography, optical spectroscopy or thermography, and MRI. All of these methods, albeit capable of high-resolution imaging, rely primarily on the detection of structural abnormalities. More recently, MRI has been used to target the cellular uptake of macrophages with the use of long circulating iron oxide particles originally developed in our laboratory. Alternatively, other plaque-associated targets have been used as biomarkers for nuclear imaging.31,32 We believe that the enzyme-sensitive beacons could have advantages as imaging agents because of their inherent built-in amplification and because they report molecular rather than anatomic abnormalities.

A number of different imaging setups can be used to detect the protease beacons and thus measure proteolytic activity.

**Figure 5.** Double staining for macrophage (Mac-3, FITC) and cathepsin B (Texas red) in aortic lesion in apoE−/− mouse with use of confocal microscopy. Original magnification ×40. a, Mac-3 (FITC). b, Cathepsin B (Texas red). c, Superimposed image in which areas in yellow represent colocalization of Mac-3 and cathepsin B immunoreactivity.

**Figure 6.** RT-PCR and Western blot analysis of cathepsin B in aortas from apoE knock-out and wild-type (WT) mice. a, Quantitative analysis of cathepsin B/β-actin cDNA ratios of aortic lesions. AA indicates abdominal aorta. b, Corresponding gel with numbers matching different lesions: 1 indicates aortic arch with lesions from apoE−/−; 2, abdominal aorta with lesions from apoE−/−; 3, abdominal aorta without lesions from WT control mice. Top panel shows cathepsin B; bottom panel, β-actin. c, Cathepsin B Western blot of different aortic segments from apoE−/− and WT mice: 1 indicates aortic arch with lesions; 2, abdominal aorta with lesions; and 3, abdominal aorta without lesions.
within atherosclerotic lesions. Depending on the desired resolution, depth penetration (micrometers versus centimeters), and application (in vitro versus in vivo), these methods include intravital microscopy (confocal or multiphoton), reflectance imaging, transillumination imaging, phased-array imaging, or FMT. Reflectance imaging, either macroscopically, as performed in the present study (Figure 1), or through catheters, would allow high-resolution imaging of plaques. Fiberoptic catheters are currently used for spectral analysis of plaques33 or angiography.34 Alternatively, we envision that FMT could become a valuable clinical tool because it is quantitative22 and because near-infrared light can penetrate parenchymal tissues up to >10 cm.35 Thus, a further combination of FMT with MRI (see Figure 1) or volume CT imaging could provide powerful new imaging tools for plaque characterization.

Despite the encouraging results of the present study, several points will require further investigation. The animal model chosen (apoE and apoE/eNOS knockout mice fed a western-type diet) only approximates human atherosclerotic disease.21 The animals routinely develop accelerated, “active,” inflammation-rich atherosclerosis, ischemic heart disease, and vascular remodeling, leading to aortic aneurysms. Because animals undergo all of these changes, it is difficult to directly assess the progression of silent to vulnerable plaques in this model. Therefore, a different regimen of diet, the use of transgenic models or other animals models, and, ultimately, clinical trials may be required to shed more light on this issue. Another issue, not addressed in the present study, is the question of which proteolytic enzyme represents the most ideal imaging biomarker in the transition of silent to vulnerable plaque. Ideally, future studies measuring expression levels of proteases in surgical specimens would be warranted.

Overall, we believe that the results of the present study have implications in understanding and imaging vulnerable plaques. The described probe armamentarium and near-infrared photon detection technology are new techniques with a potential for supplementing anatomic imaging information. Perhaps the greatest strength of this approach could be the ability to identify plaques that are most unstable or vulnerable to rupture, thus identifying high-risk patient populations.

References
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