Inflammation in Atherosclerosis

Visualizing Matrix Metalloproteinase Action in Macrophages In Vivo

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Background — Matrix metalloproteinases (MMPs) in inflamed atherosclerotic plaques may contribute to extracellular matrix remodeling and the onset of acute thrombotic complications.

Methods and Results — To test the hypothesis that optical molecular imaging with the use of an activatable near-infrared fluorescence (NIRF) probe can detect enzymatic action of MMP in atherosclerotic plaques, we used a NIRF substrate for gelatinases (MMP-2/gelatinase-A and MMP-9/gelatinase-B) in apolipoprotein E–deficient (apoE−/−) mice that consumed a high-cholesterol diet for 12 weeks and age-matched apoE+/+ mice as control. The aortas of apoE−/− mice at 24 hours after probe yielded intense NIRF signals, as detected by NIRF reflectance ex vivo, compared with negligible signals in aortas of apoE+/+ mice with/without probe administration or atherosclerotic apoE−/− aortas without probe. Gelatinase inhibitor treatment abolished NIRF signals in apoE−/− mouse aortas ex vivo. Sites of gelatinase activity visualized by NIRF colocalized with macrophage accumulation, immunoreactive MMP-2 and MMP-9, and gelatinolytic activity detected by in situ zymography. Furthermore, fluorescence molecular tomography indicated in vivo that atherosclerotic aortas of apoE−/− mice produced NIRF signals for gelatinase action, whereas aortas of apoE+/+ mice injected with the probe or apoE−/− aortas with no probe exhibited negligible NIRF signals.

Conclusions — These results suggest the feasibility of noninvasively imaging the enzymatic action of MMPs in vivo, an approach that may gain inflammatory foci in atherosclerosis, assess cardiovascular risk, and evaluate the effects of therapeutic interventions. (Circulation. 2006;114:55-62.)

Key Words: atherosclerosis ■ imaging ■ inflammation ■ macrophages ■ metalloproteinases

Inflammation in atherosclerosis contributes importantly to all stages of atherosclerosis, including the onset of acute thrombotic complications.1-3 Breakdown of extracellular matrix, a determinant of integrity of the artery, by matrix metalloproteinases (MMPs) and consequent plaque disruption can precipitate acute arterial thrombosis.4-6 Activated cells in inflamed atherosclerotic plaques (eg, macrophages, smooth muscle, and endothelial cells) produce MMPs, including gelatinases (MMP-2/gelatinase-A and MMP-9/gelatinase-B).7-14 Accumulating laboratory and clinical evidence indicates that lipid lowering may prevent acute coronary events in part by reducing inflammation, including the expression and activity of MMPs such as MMP-2 and MMP-9.5,11,12,15,16 Indeed, clinical and genetic studies correlated MMP-2/gelatinase-A and MMP-9/gelatinase-B with cardiovascular events in patients.17-23 Therefore, detection of MMP action in atherosclerotic plaques in vivo could provide a powerful tool for evaluation of vascular inflammation, for determination of individualized therapeutic strategies, and for monitoring the effects of therapeutic interventions.

The rapidly emerging strategy of molecular imaging can provide functional and biological information on physiological or pathological processes in intact organisms, whereas conventional imaging approaches such as angiography, ultrasound, magnetic resonance imaging, and computed tomography offer primarily structural information.24 In particular, an optical imaging technique that employs dedicated biocompatible fluorochromes producing near-infrared fluorescence (NIRF) signals recently has enabled visualization of cancer tissues, a useful tool not only for diagnosis but also for monitoring effects of cancer treatment.25-28 The important advantages of fluorescence imaging include the availability of signal amplification on
enzymatic activation of probes. Such activatable imaging probes conjugated with NIRF fluorochromes (eg, Cy5.5, AF680, VT680) produce very low background fluorescence, which enable hundreds-fold amplification of fluorescence after cleavage releases quenching. The NIR spectrum avoids limitations common to visible light and infrared light due to biological absorbers, hemoglobin in visible light, and water/lipids in infrared. Additionally, NIRF signals penetrate tissue up to 10 cm, thus enabling in vivo imaging. Moreover, fluorescence molecular tomography (FMT) has allowed the noninvasive detection of such signals in vivo in whole animals. Accordingly, this study tested the hypothesis that combining an activatable NIRF probe with FMT can detect inflammation in atherosclerotic plaques noninvasively in vivo following the sentinel of MMP-gelatinase activity.

Methods

Animals and Diet
Apolipoprotein E–deficient mice (apoE/−, C57BL/6; 12 weeks of age; n=19; The Jackson Laboratory, Bar Harbor, Me) consumed an atherogenic diet (semipurified chow containing 1.25% cholesterol and 0% cholate, Research Diets, Inc, New Brunswick, NJ) for 12 weeks. ApoE wild-type (apoE+/+; n=9) mice fed a normal chow (Research Diets) served as controls. All experiments conformed to a protocol approved by the Standing Committee on Animals of Harvard Medical School.

NIRF Probe for Gelatinases
The NIRF gelatinase probe was synthesized as previously described. The probe consists of a gelatinase substrate sequence, GGPRQITAG, which was previously identified from a phage library. MMP-2 or MMP-9 cleaves this probe with similar kinetics. MMP-2 or MMP-9 augments this probe’s fluorescence in vitro by ~200-fold (excitation=675 nm and emission=694 nm).

NIRF Reflectance Imaging Ex Vivo
Mouse aortas (n=19) were imaged ex vivo by NIRF reflectance imaging, as previously described. We administered the gelatinase-imaging probe (8 nmol per mouse) intravenously and excised the aortas 24 hours later. Visible light and NIRF images with a 2-minute acquisition were captured with a 12-bit monochrome CCD camera (Kodak, New Haven, Conn) equipped with an f/1.2 12.5–75-mm zoom lens and an emission long-pass filter at 700 nm (Omega Optical, Brattleboro, Vt). Validation of the specificity of the probe used a gelatinase inhibitor (MMP-2/9 Inhibitor III, H-Cys1-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys10-OH, cyclic: 1→10, 200 μmol/L, Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany). We incubated atherosclerotic aortas in DMEM at 37°C without serum for 4 hours. The aortas were then pretreated with the gelatinase inhibitor for 8 hours before further incubation with the probe (24 hours).

FMT Imaging In Vivo
We imaged animals 24 hours after intravenous administration of the NIRF probe (8 nmol per mouse). FMT noninvasively collects photons propagated through tissue at multiple projections and combines these measurements tomographically to obtain the distribution of fluorochromes in deep tissues. The detailed design and testing of the FMT imaging system built in-house and mathematical image reconstructions were described elsewhere.

Histological Validation
Dissected aortas were snap-frozen in OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned (6-μm thickness). Fresh-frozen sections were imaged with the use of an Eclipse 80i epifluorescence microscope (Nikon, Enfield, NJ) with a Cooled CCD Cascade Camera (Photometrics, Roper Scientific, Inc, Tucson, Ariz). Fluorescence images were captured through spectrally resolved channels for Cy5.5 (excitation=650 nm and emission=710 nm) and for autofluorescence (emission=480 nm and excitation=580 nm). We then performed hematoxylin and eosin staining for general morphology and immunohistochemistry for localization of immunoreactive gelatinases using rabbit polyclonal antibodies against MMP-2 and MMP-9 (Chemicon, Temecula, Calif).

In Situ Gelatin Zymography
In situ detection of gelatinolytic activity employed a quenched fluorescein-labeled gelatinase substrate, DQ-gelatin (Invitrogen, Carlsbad, Calif). Low-gelling-temperature agarose (1%, Sigma-Aldrich, St Louis, Mo) containing DQ-gelatin (0.1 mg/mL) was applied on sections of atherosclerotic aortas of apoE−/− mice injected with the NIRF gelatinase probe. Tissue sections pretreated with MMP-2/9 inhibitor III (200 μmol/L) or phenanthroline (0.1 to 1.0 mmol/L) and sections incubated with agarose gel lacking DQ-gelatin served as negative controls. After the incubation for 3 hours at room temperature, fluorescence signals elaborated from the NIRF probe and DQ-gelatin were captured through 700- and 480-nm channels, respectively. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.
### Results

#### Atherosclerotic Aortas Exhibited NIRF Signals for Gelatinase Action Ex Vivo

We used aortas of apoE<sup>−/−</sup> mice (n=6) and cholesterol-fed apoE<sup>−/−</sup> mice (n=9) to validate the NIRF probe for gelatinase action ex vivo. Visible light images showed similar extent of atherosclerotic lesion formation in all 3 aortas (top). Whereas a NIRF image of apoE<sup>−/−</sup> aortas without probe shows negligible signals, the gelatinase probe yielded substantial signals (bottom). Coincubation with the gelatinase inhibitor diminished NIRF signals in apoE<sup>−/−</sup> mouse aorta. Three independent experiments gave similar results.

#### Inhibition of Gelatinase Activity Abolished NIRF Signal in Atherosclerotic Aortas

Use of a cyclic peptide gelatinase-selective inhibitor (200 μmol/L)<sup>34</sup> in organoid culture of apoE<sup>−/−</sup> atherosclerotic aortas tested the specificity of the probe. The aortas were pretreated with the gelatinase inhibitor for 8 hours before probe administration. Visible light images showed similar atherosclerotic lesion formation in all 3 aortas (Figure 2). Although a NIRF image of apoE<sup>−/−</sup> aortas without probe showed negligible signals, the gelatinase probe provided substantial signals. Treatment with the gelatinase inhibitor markedly reduced NIRF signals in the aortas of apoE<sup>−/−</sup> mice.

#### NIRF Signals Colocalized With Macrophages Expressing MMP-2/Gelatinase-A and MMP-9/Gelatinase-B

We validated the NIRF gelatinase probe histochemically. ApoE<sup>−/−</sup> mouse aortas with negligible NIRF signal had undetectable levels of immunoreactive MMP-9 (Figure 3i). However, sites of atherosclerotic aortas of cholesterol-fed apoE<sup>−/−</sup> mice with fluorescence signals (Figure 3ii and 3iii) colocalized with MMP-9 accumulation in the intima sampled at the indicated sites (panels ii and iii). We further determined the relationship between NIRF signals and gelatinase expression in macrophage-rich and -poor plaques. Cross-sectional fluorescence images, captured through the 700-nm channel, localized NIRF signals in the macrophage-rich intima of atherosclerotic aorta of apoE<sup>−/−</sup> mice (Figure 4A, top left), where autofluorescence was barely detected through the 480-nm channel (top middle). Notably, the tunica media produced substantial signals primarily as a result of elastin autofluorescence. NIRF signals in the intima colocalized well with macrophages (Mac-3) bearing immunoreactive MMP-2/gelatinase-A and MMP-9/gelatinase-B. In contrast, we barely detected either NIRF signals or autofluorescence in the aortic intima of apoE<sup>−/−</sup> mice, which contained few if any macrophages and low gelatinase levels (Figure 4B). Furthermore, NIRF signals (700-nm channel, red) did not colocalize with intimal smooth muscle cells (identified via α-actin expression) or endothelium (Figure 5). Tissue autofluorescence
(green) was detected through the 480-nm channel and was used for orientation.

**NIRF Signals Colocalized With Gelatinolytic Activity**

In situ zymography with the use of quenched fluorescein-labeled gelatin further validated the NIRF gelatinase probe. Fluorescence signals captured through the 700-nm channel (Figure 6a) in the aortic intima of apoE−/− mice injected with the NIRF gelatinase probe colocalized gelatinolytic activity detected by zymography on the same tissue section (Figure 6b). Pretreatment with inhibitors of MMP action, phenanthroline (0.1 to 1.0 mmol/L, data not shown) and an MMP-2/9-selective inhibitor (200 μmol/L; (Figure 6c), substantially reduced fluorescence signals detected via the 480-nm channel. Agarose gel lacking fluorescein-labeled gelatin also showed lower fluorescence signals (Figure 6d).

**Atherosclerotic Aortas Exhibited NIRF Signals In Vivo**

In vivo FMT detected low NIRF signals in apoE−/− mice 24 hours after probe injection, as shown on a tomographic image superimposed by pseudocolor analysis (Figure 7, top left; n=3). An ex vivo visible light image on the aorta excised after an in vivo FMT session showed an apparently normal aorta (Figure 7, top middle). Ex vivo NIRF reflectance revealed little signal in this normal aorta of the apoE wild-type mouse (Figure 7, top right). Although cholesterol-fed apoE−/− mice without probe injection (another negative control; Figure 7, middle panels; n=3) exhibited negligible NIRF signals in vivo and ex vivo, a visible light image showed atherosclerotic plaques. Compared with these 2 control groups, atherosclerotic aortas from cholesterol-fed apoE−/− mice after probe yielded more intense NIRF signals in vivo (Figure 7, bottom panels; n=3). Ex vivo NIRF reflectance also detected NIRF signals on the aortic root and arch of apoE−/− mice.

**Discussion**

The inflammatory state of the atherosclerotic plaque, not only its size or degree of luminal stenosis, likely governs the onset of acute thrombotic complications.1–3,5 In addition to conven-
tional risk assessment and imaging modalities (eg, lipid profile, angiography), new diagnostic tools that interrogate atheroma inflammation could serve to test pathophysiological hypotheses, to identify patients at risk while the disease remains silent, and to evaluate novel therapeutic strategies. Accumulating evidence has established that increased production of various proinflammatory and thrombogenic molecules by cells in atherosclerotic plaques (eg, activated macrophages) triggers acute coronary events. However, no conventional structural imaging techniques can gauge the inflammatory state of the plaque. The development of activatable fluorescence probes that emit in the NIR spectrum, wavelengths that minimize absorption, and highly sensitive imaging methods (eg, FMT) renders noninvasive macroscopic observation of fluorescence in tissues feasible. The present study harnesses these technologies to detect MMP action, a pathophysiologically relevant index of inflammation, in mouse atherosclerotic plaques.

We detected in vivo and validated ex vivo increased gelatinase activity in mouse atherosclerotic aortas. Traditional imaging studies of atherosclerosis have evaluated anatomy and structure. Recent work has begun to probe function as well. Magnetic resonance imaging with the use of magnetic nanoparticles phagocytosed by lesional macrophages may relate to plaque inflammation. Although phagocytic activity has great relevance for microbicidal functions of leukocytes, it has little direct relevance to complications of atherosclerosis. Nuclear imaging based on fluorodeoxyglucose uptake may link to metabolic functions of phagocytes in atherosclerotic plaques. However, this approach also lacks a direct link with atherogenesis and its complications.

In contrast, previous studies including our own have established a critical role for MMPs, including MMP-2/gelatinase-A and MMP-9/gelatinase-B, in various forms of cardiovascular remodeling such as progression and acute complications of atherosclerosis, restenosis after vascular intervention, aneurysm formation, and valve disease. Gough et al recently demonstrated that enforced expression of an active form of MMP-9/gelatinase-B in macrophages induces disruption of atherosclerotic plaques in apoE−/− mice. After initial cleavage by MMP-collagenases, gelatinases can degrade fibrillar collagen, a determinant of integrity of the artery. MMP-2/gelatinase-A itself may degrade fibrillar collagen. MMP-9/gelatinase-B has various substrates (eg, interleukin-1β precursor, tissue factor pathway inhibitor) and therefore may contribute to other aspects of the pathogenesis of vascular inflammation and thrombosis. A number of previous studies also established the contribution of gelatinases to various aspects of other

Figure 5. Macrophages constitute the major source of NIRF signals for gelatinase action in mouse atheromata. NIRF signals (700 nm, red) localized with immunoreactive macrophages (Mac-3), but not smooth muscle cells (SMC) (α-actin) or endothelium, in the atherosclerotic plaque of apoE−/− mice. Autofluorescence signals (480 nm, green) were merged for better orientation of the tissue. Three independent experiments provided similar results.

Figure 6. In situ zymography correlated NIRF signals with gelatinolytic activity. Fluorescence elaborated from the NIRF gelatinase probe (700 nm; a) colocalized with signals detected by zymography with the use of agarose gel containing quenched fluorescein-labeled gelatin (480 nm; b) in the atherosclerotic plaque of apoE−/− mice. Gelatinase inhibition by an MMP-2/9-selective inhibitor (c) and the use of agarose gel lacking fluorescein-conjugated gelatin (d) reduced signals in the intima. These results represent 3 independent experiments that provided similar results.
disease processes such as rheumatoid arthritis, cancer, and angiogenesis.\textsuperscript{49,50} Use of an activatable NIRF probe after experimental acute myocardial infarction in mice recently demonstrated ex vivo the feasibility of gelatinase activity detection.\textsuperscript{31}

The present study shows in vivo that a similar strategy can detect MMP-2/gelatinase-A and MMP-9/gelatinase-B action noninvasively in macrophage-rich atherosclerotic aortas in hypercholesterolemic mice. Validation studies further demonstrated that signals elaborated from the NIRF gelatinase probe colocalized with macrophages expressing MMP-2 and MMP-9 (Figures 3 to 5). In contrast, more fibrous plaques that contained few macrophages showed negligible NIRF signals (Figure 4B). Lipid lowering reduces macrophage accumulation in patients and experimental animals.\textsuperscript{5,11,12,15,16} Therefore, our data suggest that gelatinase imaging not only identifies inflamed plaques but also may reflect differences in inflammatory burden among individual plaques. Clinical extensions of this approach could involve examination of superficial arteries (eg, carotid) noninvasively or of deeper arteries by using a catheter-based intravascular detection platform in development.

The present study builds on more than a decade of fundamental research on the inflammatory pathways that prevail in the atherosclerotic plaque and promote its clinical expression.\textsuperscript{1-5} Gauging inflammation within atherosclerotic plaques with the use of MMP activity as a pathobiologically validated indicator may have several clinical applications, including the assessment of the inflammatory activity, not merely the anatomy, of atherosclerotic disease in individual patients. This indicator may also provide a research tool to assess the relationships between plaque inflammation and clinical events and outcomes. Finally, noninvasive imaging of inflammation directly implicated in plaque pathophysiology may furnish a much needed biomarker or surrogate end point for the evaluation of novel therapies that target this process.

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Disclosures

None.

References

Enzymes specialized in breakdown of extracellular matrix known as matrix metalloproteinases (MMPs) participate in various aspects of tissue remodeling. Considerable data indicate that degradation of arterial collagen by MMPs favors disruption of atherosclerotic plaques and consequent arterial thrombosis. Inflammatory mediators elicit overexpression of MMPs by vascular cells and macrophages. Recent advances in molecular and functional imaging join detection of biological processes to the anatomic information available from traditional imaging modalities. This study explored the feasibility and validation of in vivo imaging of inflammation in atherosclerotic lesions by visualization of MMP activity in mice. Cleavage by MMP gelatinases (MMP-2 and MMP-9) rendered the used agent intensely fluorescent in the near-infrared range in a specific and time-dependent manner in vitro. Use of this probe revealed signals in the explanted atherosclerotic aortas of hypercholesterolemic apolipoprotein E−deficient mice that correlated with immunolocalized MMP-2 and MMP-9. Administration of this MMP probe to intact atherosclerotic mice yielded fluorescent signal in affected arteries detected noninvasively by tomographic techniques. Near-infrared fluorescent probes such as the one used in this study should permit noninvasive imaging of inflammation in superficial arteries in humans and in deeper arteries by catheter-based detectors. Imaging of inflammatory foci in atheromata will permit testing of pathophysiological hypotheses in vivo and could serve as an intermediate end point based on biological function in addition to anatomy in the evaluation of novel therapies that target inflammation in atherosclerosis.
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