Optical Visualization of Cathepsin K Activity in Atherosclerosis With a Novel, Protease-Activatable Fluorescence Sensor

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Background—Cathepsin K (CatK), a potent elastinolytic and collagenolytic cysteine protease, likely participates in the evolution and destabilization of atherosclerotic plaques. To assess better the biology of CatK activity in vivo, we developed a novel near-infrared fluorescence (NIRF) probe for imaging of CatK and evaluated it in mouse and human atherosclerosis.

Methods and Results—The NIRF imaging agent consists of the CatK peptide substrate GHPGGPGKC-NH₂ linked to an activatable fluorogenic polymer. In vitro, CatK produced a 2- to 14-fold activation of the agent over other cysteine and matrix metalloproteinases (P<0.0001), as well as a >8-fold activation over a control imaging agent (P<0.001). Optical imaging of atheroma revealed >100% NIRF signal increases in apolipoprotein E⁻/⁻ mice in vivo (n=13; P<0.05, CatK imaging agent versus control agent) and in human carotid endarterectomy specimens ex vivo (n=14; P<0.05). Fluorescence microscopy of plaque sections demonstrated that enzymatically active CatK (positive NIRF signal) localized primarily in the vicinity of CatK-positive macrophages. Augmented NIRF signal (reflecting CatK activity) colocalized with disrupted elastin fibers within the media underlying plaques.

Conclusions—Use of this novel protease-activatable NIRF agent for optical imaging in vivo demonstrated preferential localization of enzymatically active CatK to macrophages, consistent with their known greater elastinolytic capabilities compared with smooth muscle cells. Augmented CatK proteolysis in atheromata further links CatK to vascular remodeling and plaque vulnerability. (Circulation. 2007;115:2292-2298.)

Key Words: atherosclerosis • cathepsin K • fluorescence • imaging • inflammation

Through degradation of the extracellular matrix, proteases contribute importantly to the progression and complications of atherosclerosis.¹⁻⁴ Several families of proteases participate in atherogenesis, including matrix metalloproteinases (MMPs), cysteine proteases, and serine proteases. Recently, experimental studies have demonstrated a role for the lysosomal cysteine protease cathepsin K (CatK) in atherosclerosis.⁵⁻⁶ CatK, one of the most potent elastases yet identified,⁷ localizes in rupture-prone areas such as the fibrous cap and plaque shoulders, as well as at the actual site of plaque ruptures. The matrix-degrading protease CatK may thus contribute to plaque destabilization, the key event underlying acute coronary syndromes, and its inhibition might prove useful for treating atherosclerosis. Therefore, imaging of CatK activity in vivo has considerable biological and clinical potential.

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The present study investigated a CatK-sensitive protease-activatable agent for optical imaging of atherosclerosis. Quenched at baseline, these protease-activatable substrates fluoresce brightly after cleavage by their target enzymes (reviewed elsewhere⁸). The dequenched fluorochromes emit in the near-infrared (NIR) range an ideal window for in vivo imaging as a result of lower autofluorescence and deeper photon penetration through tissue and can be detected by a number of in vivo fluorescence imaging systems.⁹ Importantly, these protease-activatable sensors directly report the in

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vivo activity of the target enzyme classes, providing information complementary to immunolocalization in tissue sections. In addition, these sensors are moving rapidly toward clinical translation, with the potential for tomographic, catheter-based, and microscopic detection.

This study investigated the ability to image CatK activity in mouse atheroma in vivo and in human endarterectomy specimens, the cellular source of enzymatically active CatK in regions of vascular remodeling in atherosclerosis, and the relationship of CatK activity with rupture-prone and remodeled areas of atherosclerotic lesions.

Methods

Synthesis of the CatK-Sensitive and Control NIR Fluorescence Imaging Agents

Please see the online Data Supplement for additional details.

The CatK imaging agent consists of a polymer backbone derivatized with multiple copies of a CatK-cleavable peptide substrate containing an NIR fluorochrome, resulting in strong intramolecular quenching at baseline. After enzymatic cleavage, the fluorochromes separate, resulting in substantial amplification of the NIR fluorescence signal.

Protected graft copolymers (PGC) consisting of a poly-L-lysine (N,N-dimethylformamide), and methoxy poly(ethylene glycol) side chains were prepared as described.

The CatK-FITC and d-CatK-FITC peptide substrates were attached to PGC and d-PGC, respectively, similar to previously reported probes.

The number of peptide chains per PGC molecule was estimated from FITC absorption at 494 nm.

Protected graft copolymers (PGC) consisting of a poly-L-lysine (N,N-dimethylformamide), and methoxy poly(ethylene glycol) side chains were prepared as described.

Conjugation of FITC-Labeled Peptide Substrates to Protected Graft Copolymer Backbones

Please see the online Data Supplement for additional details.

Protected graft copolymers (PGC) consisting of a poly-L-lysine (N,N-dimethylformamide), and methoxy poly(ethylene glycol) side chains were prepared as described.

NIRF Fluorochrome Conjugation of Imaging Agents

To render the agents fluorescent in the NIR channel, CatK-FITC-PGC was first concentrated with a Micron YM–50 filter to 150 μL volume and then reacted for 2 hours at room temperature with excess Cy 5.5-NHS (Amersham, Australia) in the presence of 0.1 mol/L NaHCO3. The same reaction was performed with the control d-CatK-FITC-d-PGC. The NIR agents CatK-FITC-PGC-Cy5.5 (CatK imaging agent) and d-CatK-FITC-d-PGC-Cy5.5 (control agent) were recovered by filtration with a Micron YM–50 filter. To remove unconjugated Cy5.5, the dark green polymers were washed extensively with deionized water until the recovered filtrate appeared colorless.

In Vitro Enzymatic Studies

Relative protease selectivity was determined with human liver CatB, human recombinant CatL, human recombinant CatK, human recombinant MMP-2, and human recombinant MMP-9 (Calbiochem, La Jolla, Calif). In each well, the CatK agent (0.1 nmol) was incubated with 50 pmol protease in its optimal buffer (200 μL) condition, and...
the fluorescence signal was measured for 15 hours. Cathepsin activation was performed in 100 mmol/L PBS, 2 mmol/L EDTA, and 2 mmol/L DTT, pH 6.0, and MMP activation was done in 25 mmol/L HEPES and 5 mmol/L CaCl₂, pH 7.3. Generated Cy5.5 fluorescence over time was measured with a plate reader (GENios Pro plate reader, Tecan, Durham, NC) using excitation at 670 nm and emission at 720 nm. Experiments were run in triplicate.

To compare relative activation of the CatK and control NIRF imaging agents, 0.21 nmol (60 μL) of the CatK agent (CatK-FITC-PGC-Cy5.5) or the control agent (d-CatK-FITC-d-PGC-Cy5.5) was incubated with 3 μL (100 μL) of His-tag human recombinant CatK at 37°C for 15 hours. The total volume in each well was adjusted to 300 μL with 100 mmol/L phosphate buffer solution (PBS), 2 mmol/L EDTA, and 2 mmol/L DTT, pH 6.0. Equal amounts of the polymeric probes also were incubated with PBS only. Experiments were run in triplicate.

**NIRF Imaging of In Vivo CatK Activity in Experimental Murine Atherosclerosis**

Apolipoprotein E–deficient (ApoE⁻) mice (n = 13; Jackson Laboratories, Bar Harbor, Maine) at 10 weeks of age consumed a high-cholesterol diet (0.2% cholesterol, Harlan Teklad, Madison, WI) for an additional 20 weeks. Under inhalational anesthesia (1% to 2% isoflurane plus 2 L O₂), mice received a tail vein injection of 5 nmol CatK agent (n = 7) or control agent (n = 6) diluted to a volume of 150 μL in PBS. After 24 hours, animals were euthanized with CO₂ and perfused with saline, and their aortas were excised. Aortas next underwent fluorescence reflectance imaging (FRI) using a custom-built apparatus as previously detailed. White-light and Cy5.5 NIRF images (4 averaged 1-minute acquisitions) were acquired. The peak aortic NIRF signal was determined as the mean signal intensities (SIs) from regions of interest (52 pixels) placed on the highest-signal areas. The peak plaque target-to-background ratio (TBR) was calculated as follows: TBR = SI (plaque) / SI (normal adjacent aorta).

After imaging, aortas were frozen in optical coherence tomography compound (Sakura Finetek, Torrance, Calif) and windowed with Adobe Photoshop software (version 5.5, Adobe, San Jose, Calif) for histopathological analysis. All animal protocols were approved by the Subcommittee on Research Animal Care at our institution.

**Intravital Fluorescence Microscopy**

To image CatK activity in vivo, a subset of injected animals (n = 6; 3 CatK imaging agent, 3 control) underwent nonsurvival intravital fluorescence microscopy (IVFM) of atheroma as described recently. Briefly, anesthetized mice underwent surgical exposure of the right carotid artery, with placement of a fluorescent phantom directly underneath the atherosclerotic vessel. IVFM was performed with a multichannel prototypical laser scanning microscope (IV 100, Olympus Corp, Tokyo, Japan) with 3 laser lines (488-, 633-, and 748-nm excitation) and optimized objectives designed for invasive imaging. A vascular NIRF agent (Angiosense750, VisEN Medical, Woburn, Mass) was coinjected to provide a vascular angiogram outlining the plaque. The in-plane resolution for the X/Y objective used was 13 × 13 μm, and z stacks were obtained at 10-μm steps. Image analysis was performed by compiling the z stack into a 2-dimensional projection image (ImageJ, version 1.36b, Bethesda, Md). Regions of interest were circled within the plaque (as defined by the 680 nm channel; band-pass emission filter, 660 to 730 nm) and the adjacent vascular space (as defined by the AF750 channel; long-pass emission filter, 770 nm). The plaque TBR was calculated as above.

**NIRF Imaging of CatK Activity in Human Atherosclerotic Specimens Ex Vivo**

Human carotid endarterectomy specimens (n = 14) were obtained with an Institutional Review Board–approved protocol. Freshly resected carotid specimens were washed with PBS and incubated in Dulbecco’s modified Eagle’s medium at pH 7.4. Next, 2 mmol CatK agent (n = 8), control agent (n = 5), or PBS (n = 3) was added to the specimen and then incubated in 5% CO₂ at 37°C. At 1, 6, and 24 hours, FRI was performed using a prototypical reflectance imaging system (BonSAI, Siemens, Berlin, Germany). White-light, FITC-channel (100-ms acquisition), and Cy5.5 NIRF-channel (500-ms acquisition) images were acquired. The mean NIRF signal of the entire carotid specimen was measured using the custom-built software package CMIR Image. Specimens were then frozen in optical coherence tomography compound for histopathological analysis.

**Fluorescence Microscopy**

Cryosections (6 μm) were obtained from the fresh-frozen mouse aortas and human carotid endarterectomy specimens. Fluorescence microscopy was performed with an inverted epifluorescence microscope (Zeiss Axiovert, Thornwood, NY) to visualize the distribution of Cy5.5 fluorescence in plaque sections (exposure time, 1.0 seconds). Adjacent sections were then stained with antibodies against CatK, macrophages, and smooth muscle α-actin as described below.

**Immunohistochemistry**

Serial fresh-frozen sections of human or mouse atheromata were fixed with 4% paraformaldehyde for 5 minutes. Immunohistochemistry was performed using the avidin-biotin-peroxidase method. Briefly, sections treated with 0.3% hydrogen peroxide were incubated for 60 minutes with primary or isotype control antibodies, followed by respective biotinylated secondary antibody. The reaction was visualized with 3-amin-9-ethylcarbazole, or AEC, substrate and counterstained with Harris hematoxylin solution. Cells in human atheroma sections were identified with monoclonal CatK-specific antibody, anti-CD68 for macrophages, and smooth muscle α-actin for smooth muscle cells. Cells in mouse atheroma sections were identified with anti-Mac3 for macrophages and smooth muscle α-actin for smooth muscle cells (BD PharMingen, San Diego, Calif).

Staining for CatK used a rabbit polyclonal antibody. Tissue sections were viewed with a microscope (Nikon Eclipse 50i, Tokyo, Japan), and images were digitally captured with a charge-coupled device–SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, Mich) and windowed with Adobe Photoshop software (version 5.5, Adobe, San Jose, Calif).

**Statistical Analysis**

Data are presented as mean ± SD. Student t test was used to compare the mean NIRF signal intensities for murine FRI and IVFM studies. For differences between groups in the in vitro and human specimen experiments, a 1-way ANOVA, followed by a post-hoc Tukey’s test for multiple comparisons, was used. A value of P < 0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**CatK Enzyme Highly Activates the CatK NIRF Imaging Agent In Vitro**

We first compared the activation of the CatK imaging agent by CatK compared with other proteases. In vitro, CatK enzyme activated the agent >2-fold compared with CatB and CatL, and >14-fold compared with MMP-2 and MMP-9 (P < 0.001; Figure 1B). Next, we compared the in vitro fluorescence activation of the CatK protease-activatable and control NIRF imaging agents with and without CatK enzyme addition. Serial fluorescence measurements over time demonstrated that only the CatK agent plus enzyme group substantially amplified its fluorescence signal (average of 3 experiments; Figure 1C). Incubation of the CatK agent with the CatK enzyme increased the NIRF signal 8.97 ± 0.06-fold over baseline. Omission of the enzyme yielded scant NIRF signal amplification over baseline (0.92 ± 0.00), as did incubation of the control agent with (1.52 ± 0.02) or without
was concentrated in the aortic root and aortic arch, well-visible in all ApoE
negative Cy5.5 NIRF signal (Figure 2A). Signal enhancement
as filling defects within the vascular space (Figure 3B). Control agent had minimal NIRF signal enhancement, appear-
strong focal NIRF signal in the Cy5.5 channel was present
the CatK agent group, strong focal signal was present within the aortic root, aortic
mineral CatK activity macroscopically, we performed FRI 24 hours
after intravenous agent injection. Atherosclerotic aortas of
mice injected with the CatK imaging agent (Figure 2A). Signal enhancement
in the aortic root and aortic arch, well-known sites of atherosclerosis in ApoE
mice. In contrast, atherosclerotic animals injected with the control agent
focal signal enhancement in areas of elastin fiber disruption at the
NIRF microscopy of murine plaque sections revealed focal signal enhancement in areas of elastin fiber disruption at the
intimal-medial border (Figure 4A) and intimal-luminal inter-
facing consistent with earlier studies. However, CatK de-
tected by immunohistochemistry had a broader signal profile than that of the CatK NIRF agent, suggesting that only a
subset of immunoreactive CatK was enzymatically active
(Figure 4B). Furthermore, the plaque NIRF signal enhance-
ment preferentially colocalized with macrophages (Figure
rather than medial smooth muscle cells that were α-actin
positive (Figure 4D). In contrast, atherosclerotic plaques
retrieved from mice injected with the control imaging agent
demonstrated minimal NIRF signal within the plaque, despite
the presence immunoreactive CatK (Figure 4E and 4F).
Similar levels of NIRF were noted within the medial fibers in

**Figure 2.** FRI of aortic plaques from ApoE
to image CatK activity in vivo used IVFM of carotid arteries
in anesthetized ApoE
carotid arterioles were visible in all ApoE
twenty-four hours after CatK imaging agent injection
and immediately after coinjection of a spectrally distinct
multichannel high-resolution IVFM was performed. In mice injected with the CatK imaging agent, strong focal NIRF signal in the Cy5.5 channel was present
within the plaque, indicative of CatK activity (green; Figure
3A). The focal plaque signal was outlined and confirmed to be
intravascular by use of the vascular agent (red) that
provided an angiogram through in vivo blood flow. In
contrast, carotid plaques of ApoE
demonstrating greater plaque TBRs (E) in the CatK vs control
group (*P<0.05). Projection images processed and windowed
identically. Scale bar=250 μm.

**Figure 3.** In vivo imaging of CatK activity in carotid atheroscle-
rotic plaques of ApoE
tact agent compared with the control agent, with a 174% increase in the plaque TBR (3.5±1.4 versus 1.3±0.2; 
P<0.05; Figure 3C through 3E).

**CatK Activity Is Increased in Areas of Elastin Fiber Disruption and Colocalizes With Macrophages**

Significant pulsation artifacts were not observed with the 4× magnification objective used. Projection images from the z
stacks demonstrated greater overall signal with the CatK imaging agent compared with the control agent, with a 174%
increase in the plaque TBR (3.5±1.4 versus 1.3±0.2; 
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**In Vivo Imaging of CatK Activity in Atherosclerosis**

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positive (Figure 4D). In contrast, atherosclerotic plaques
retrieved from mice injected with the control imaging agent
demonstrated minimal NIRF signal within the plaque, despite
the presence immunoreactive CatK (Figure 4E and 4F).
Similar levels of NIRF were noted within the medial fibers in
lesions from both imaging agent groups, presumably because of elastin autofluorescence.

**Imaging of CatK Activity in Human Atherosclerosis**

To determine the detectability of CatK activity in human atherosclerotic specimens, fresh carotid atheroma specimens were incubated with imaging agents or buffer and then underwent serial FRI. In the active group, strong plaque NIRF signal evolved over 24 hours compared with the control group (Figure 5A and 5B). The autofluorescence signal (FITC channel) remained similar over time. The plaque NIRF signal at 24 hours was highest in the CatK imaging agent group (127 ± 56.7 arbitrary units [au]; control agent, 36 ± 8.0 au; PBS, 9.5 ± 6.0 au; P < 0.05, CatK versus each of the other groups; Figure 5C). No significant difference was present in the FITC-channel signal among the groups (P > 0.05). NIRF images obtained 1, 6, and 24 hours after agent incubation. The NIRF signal strongly increased over time, whereas the FITC signal (autofluorescence) remained unchanged. B, In a carotid sample incubated with the control agent, minimal NIRF signal and signal evolution are present. C and D, Quantification of the fluorescence signal revealed greater NIRF signal in the CatK group (*P*<0.05 vs other groups), with no significant difference in the FITC-channel signal among the groups (P > 0.05). NIRF images were windowed identically. Scale bar = 500 μm.

**Discussion**

The lysosomal endopeptidase CatK (EC 3.4.22.38) has potent elastinolytic and collagenolytic activity and likely contributes to the degradation of the extracellular matrix of atherosclerotic lesions. To understand further its role in atherosclerosis, we developed and investigated a novel NIRF sensor to image CatK activity in vivo.

The protease-activatable NIRF agent was engineered to be sensitive and specific to CatK cleavage by derivatizing the lysine ε-amino groups of the graft copolymer with the peptide substrate Gly-His-Pro-Gly-Pro-Gln-Gly-Lys-Cys-NH₂ (GHPGPQGKC; Figure 1). The substrate containing a key proline residue at P2²₀ has been reported to have high specificity for CatK and to resist proteolysis by CatB, CatL, CatS, CatF, CatH, and CatV, as well as the serine proteases CatG, chymotrypsin, and leukocyte elastase. In addition, fibroblasts from CatK-deficient mice do not cleave this peptide. In vitro, human CatK preferentially amplified the NIRF signal of the CatK agent compared with other cysteine proteases or MMPs (Figure 1). Furthermore, the control imaging agent produced considerably less NIRF signal amplification by CatK, demonstrating that the increased NIRF signal in the CatK group resulted from proteolytic cleavage of the peptide substrate by CatK.

In murine atherosclerotic vessels, the CatK-activatable agent provided strong (175% greater than control) NIRF signal enhancement of atheroma in vivo (Figure 3). Cleavage of the human peptide substrate by murine CatK was anticipated because of the high degree of conservation of the amino acid sequence among species. IVFM using a multiwavelength laser scanning fluorescence microscope (13×13 μm in-plane resolution) allowed discrete identification of CatK-

**Figure 4.** Correlative fluorescence microscopy of a representative atherosclerotic plaque section from an ApoE−/− mouse injected with the CatK agent. A, Focal NIRF signal was found at the plaque shoulders and intimal-medial border and showed good colocalization with CatK detected by immunohistochemistry (B; ×400). However, the NIRF signal reflective of CatK activity was more restricted than the immunohistochemical stain depicting CatK presence. The NIRF signal colocalized preferentially with macrophages (mac; C; ×400) rather than smooth muscle cells (SMC; D; ×400), suggesting that the enzymatically active CatK in atheroma was primarily associated with macrophages (arrowheads). E, In comparison, a plaque from the control imaging agent group showed minimal NIRF signal in a plaque section containing immunoreactive CatK (F). NIRF images were processed and windowed identically. Scale bar = 50 μm.

**Figure 5.** Optical imaging of CatK activity in human carotid atherosclerotic lesions. Resected endarterectomy specimens were incubated with 2 nmol CatK imaging agent, control agent, or buffer (PBS). A, Light image (left), serial NIRF-channel images (top), and FITC-channel images (bottom) of a carotid specimen incubated with the CatK probe. Fluorescence images were obtained 1, 6, and 24 hours after agent incubation. The NIRF signal strongly increased over time, whereas the FITC signal (autofluorescence) remained unchanged. B, In a carotid sample incubated with the control agent, minimal NIRF signal and signal evolution are present. C and D, Quantification of the fluorescence signal revealed greater NIRF signal in the CatK group (*P*<0.05 vs other groups), with no significant difference in the FITC-channel signal among the groups (P > 0.05). NIRF images were windowed identically. Scale bar = 500 μm.
positive carotid atheroma and the vessel lumen. With further surgical refinements, the current imaging methodology could allow serial assessments in vivo of CatK activity during therapeutic manipulations.

The present study offers insight into the cellular origin of enzymatically active CatK in atherosclerosis and emphasizes the difference between methods used to sense enzyme activity (eg, protease-activatable imaging strategies) and those that report on enzyme presence (eg, antibody-based methods such as immunohistochemistry and protein blotting). Currently available antibodies do not distinguish active CatK from zymogen precursors that lack proteolytic capacity. The present study noted strong CatK immunoreactivity in both macrophages and smooth muscle cells in atheromata, similar to earlier studies. However, we found that CatK activity, reflected by increased NIRF signal, localized nearly exclusively in the vicinity of plaque macrophages rather than smooth muscle cells (Figure 4). This finding extends finding from earlier in vitro studies demonstrating that macrophages possess a 10-fold-greater elastinolytic capacity than smooth muscle cells. In addition, macrophages in particular can secrete CatK into the extracellular space, where it remains proteolytically active. Thus, the present investigation suggests that macrophages furnish most of the enzymatically active CatK in atherosclerotic lesions.

Analysis of atherosclerotic plaques demonstrated that CatK activity colocalized in areas of disrupted elastin fiber integrity at the plaque intima-media interface (Figure 4). In addition to the potential of CatK to contribute to plaque rupture via degradation of the fibrous cap, this finding further relates CatK to plaque remodeling because its elastinolytic actions could promote lesional expansion, including compensatory remodeling. Positive (or expansive) remodeling, the expansion of the internal elastic lamina area with preservation of the lumen, typifies early plaque growth.

Furthermore, positive remodeling characterizes plaques with greater inflammation and lipid content and thus can distinguish plaques at higher risk of rupture. Moreover, as wall tension varies directly with arterial radius by the Laplace relationship, lesion expansion can promote biomechanical conditions that would favor plaque disruption.

The NIRF agent also permitted sensing of CatK activity in human atherosclerotic lesions, producing a 3.5- and 13-fold-greater signal than the control agent and PBS group (autofluorescence), respectively (Figures 5 and 6). Microscopy showed colocalization of the NIRF signal with CatK and CD68-positive macrophages, demonstrating the ability of the agent to image CatK activity in human atherosclerosis. This agent thus adds to the armamentarium of molecular imaging agents designed to detect inflammation in atherosclerosis (reviewed elsewhere). Furthermore, the CatK agent is based on a similar protease-activatable agent planned for clinical trials and thus may ultimately emerge into a clinical agent for imaging of inflammation in atherosclerosis. From an imaging perspective, NIRF visualization of CatK activity in atherosclerotic lesions of patients may be possible with catheter-based detectors, intraoperative systems, and clinically evolving reflectance and tomographic platforms.

In summary, the present investigation shows the feasibility of visualizing CatK activity in vivo in atherosclerosis. Augmented CatK proteolysis in atherosclerosis further links CatK to adverse vascular remodeling. Additional applications of the CatK-activatable imaging agent could include disorders of bone remodeling, Gaucher’s disease, amyloidosis, thyroid function, prostate cancer, and lung fibrosis.

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References
5. Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P. Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their pro-
13. Pande AN, Kohler RH, Aikawa E, Weissleder R, Jaffer FA. Detection of macro-
15. Bogdanov AA Jr, Martin C, Bogdanova AV, Brady TJ, Weissleder R. An adduct of cis-diaminedichloroplatinum(II) and polyethylene glycol poly(L-lysine) 
21. Reddy VV, Zhang QY, Weiss SJ. Pericellular mobilization of the tissue-
destructive cysteine proteases, cathepsins B, L, and S, by human monocyte-
23. Glagow S, Weisenberg E, Zaitsev CK, Stankanavicius R, Kolettis GJ Compen-
27. Jaffer FA, Libby P, Weissleder R. Molecular and cellular imaging of atheroscle-
29. Desai ND, Miwa S, Kodama D, Cohen G, Christakis GT, Goldman BS, Baer-

**CLINICAL PERSPECTIVE**

The potent elastase cathepsin K likely participates in atherosclerotic plaque remodeling. Here, we establish an optical imaging method to detect cathepsin K enzymatic activity in vivo using near-infrared fluorescence imaging technology. Using a novel protease-activatable imaging agent that fluoresces intensely after cathepsin K cleavage, we show that mouse atherosclerotic plaques possess abundant cathepsin K activity as detected by intravital microscopy, ex vivo fluorescence reflectance imaging, and fluorescence microscopy. Histopathological analyses of lesions identified macrophages as the primary cellular source of enzymatically active cathepsin K, corroborating prior in vitro studies. Augmented cathepsin K activity localized in areas of medial disruption, compatible with a role for cathepsin K in adverse vascular remodeling (Glagow effect), a characteristic of plaques that have caused thrombosis. With the development of intravascular and tomographic fluorescence imaging platforms, the protease-activatable agent could enable in vivo imaging of cathepsin K activity in human atherosclerosis.
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