Protease Imaging of Human Atheromata Captures Molecular Information of Atherosclerosis, Complementing Anatomic Imaging

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Objective—There is hope that molecular imaging can identify vulnerable atherosclerotic plaques. However, there is a paucity of clinical translational data to guide the future development of this field. Here, we cross-correlate cathepsin-B or matrix metalloproteinase-2/-9 molecular optical imaging data of human atheromata or emboli with conventional imaging data, clinical data, and histopathologic data.

Methods and Results—Fifty-two patients undergoing carotid endarterectomy (41 atheromata) or carotid stenting (15 captured emboli) were studied with protease-activatable imaging probes. We show that protease-related fluorescent signal in carotid atheromata or in emboli closely reflects the pathophysiologic alterations of plaque inflammation and statin-mediated therapeutic effects on plaque inflammation. Inflammation-related fluorescent signal was observed in the carotid bifurcation area and around ulcer-hemorrhagic lesions. Pathologically proven unstable plaques had high cathepsin-B–related fluorescent signal. The distribution patterns of the mean cathepsin-B imaging signals showed a difference between the symptomatic vs asymptomatic plaque groups. However, the degree of carotid stenosis or ultrasonographic echodensity was weakly correlated with the inflammatory proteolytic enzyme-related signal, suggesting that molecular imaging yields complimentary new information not available to conventional imaging.

Conclusion—These results could justify and facilitate clinical trials to evaluate the use of protease-sensing molecular optical imaging in human atherosclerosis patients. (Arterioscler Thromb Vasc Biol. 2010;30:449-456.)

Key Words: atherosclerosis ■ cathepsin-B ■ molecular imaging ■ protease ■ structural imaging

Conventional imaging approaches such as angiography and ultrasound offer primarily structural information and yields limited data on plaque stability. The degree of carotid stenosis, as determined by structural imaging, is currently the most important therapeutic parameter in deciding on vascular intervention in addition to medical treatment.1 There is hope that the emerging technologies of molecular imaging could provide a window of insight into the underlying molecular processes that give rise to plaque rupture.2–4

Vulnerable plaques are characterized by the presence of inflammatory mediators and proteolytic enzymes, such as cathepsins and matrix metalloproteinases, which disturb the structural integrity of atheromatous plaques and consequently provoke plaque rupture to expose the lipid-rich plaque interior to thrombin-activating blood cascades.5–7 It is the presence of these molecular species that distinguishes stable atheromatous lesions from unstable ones. We chose to leverage these molecular differences by devising imaging agents to probe for these enzymes.

We and others2,3,8,9 have developed protease-sensing near-infrared fluorescent (NIRF) molecular imaging agents that are optically silent at injection because of auto-quenching between closely spaced fluorochromes. After enzyme-specific protease-mediated cleavage, fluorochromes are dequenced and become brightly fluorescent.2,3,8,9 This technology has potential clinical applicability when combined with an intraoperative NIRF imaging system or fluorescence-sensing catheter-based system,4 as shown by multiple preclinical studies. Because NIR photons may travel up to 5 cm deep into the body, noninvasive fluorescent tomography systems may eventually detect NIRF signals from human carotid atheromata.10,11

However, a wide translational gap12 exists between the laboratory and atherosclerosis clinic. In particular, there is no prospective study comparing NIRF molecular imaging data...
with clinical data. We undertook the present study to partially bridge this gap and generate data that might be useful in the justification and design of future human trials of cathepsin-B (CatB) and matrix metalloproteinase (MMP)-2/-9 based NIRF imaging. In this prospective study, we estimate the potential clinical efficacy of these agents by cross-correlating molecular imaging data derived from patient tissues of either carotid endarterectomy or stenting with clinical data and histopathology data.

**Materials and Methods**

This study was approved by the Institutional Review Boards of the Dongguk University Ilsan Hospital and Asan Medical Center, Korea. A full description of the methods used is available (please see http://atvb.ahajournals.org).

**Patients**

From July 2006 to June 2008, 52 patients with carotid stenosis were enrolled in this study (41 men, 11 women; age±SD, 67.0±9.0 years). Thirty-seven patients were treated with carotid endarterectomy (CatB) and matrix metalloproteinase (MMP)-2/-9 activatable NIRF imaging. In the present study, we estimate the potential clinical efficacy of these agents by cross-correlating molecular imaging data derived from patient tissues of either carotid endarterectomy or stenting with clinical data and histopathology data.

**Conventional Imaging-Based and Molecular Imaging-Based Plaque Characterization**

Sonographic plaque characterization was performed to obtain plaque stenosis estimates (percentage reduction of diameter), classify the degree of carotid stenosis (normal, <50%, 50%–69%, ≥70% stenosis, or near occlusion), measure overall plaque echodensity with computer assistance, and type carotid plaque based on ultrasonic heterogeneity or echolucency. Mechanism of cerebral infarction was determined as previously described. Then, plaques that had caused a recent ipsilateral embolic stroke within 1 month were classified as symptomatic plaques. The fresh carotid specimen were washed with normal saline and tissue-cultured in DMEM (1 mL) with the CatB probe (0.67 nmol). Before and 2 hours after the incubation, NIRF reflectance imaging was performed, and then the specimens were paraffin-embedded. The emboli imaging was performed to estimate potential clinical usefulness of in vivo human CatB imaging by seeing if the vulnerable portion of a plaque, whose matrix is fragile enough to be easily separated from the plaque body and to be embolized to cause stroke in association with the procedure, would have strong proteolytic enzyme activity.

**Human Carotid Atheroma and Emboli**

Atherosclerotic plaques were obtained at carotid endarterectomy. The fresh carotid specimen were washed with normal saline and tissue-cultured in DMEM (3 mL) with either 2 nmol CatB-activatable probe (ProSense-680; Visen Medical) or 3 nmol MMP-2/-9-activatable probe (synthesized as described previously with some modification; please see http://atvb.ahajournals.org for further details with probe characterization) in 37°C CO2 incubator. For control imaging, DMEM without the probes was used. Before and 24 hours after the incubation, molecular optical imaging was performed using a NIRF imaging machine with a charge-coupled device camera (CoolSnap-EZ; Roper Scientific).

In the angioplasty group, emboli dislodged from atherosclerotic plaques were obtained from the protection device. They were washed with normal saline and tissue-cultured in DMEM (1 mL) with the CatB probe (0.67 nmol). Before and 2 hours after the incubation, NIRF reflectance imaging was performed, and then the specimens were paraffin-embedded. The emboli imaging was performed to estimate potential clinical usefulness of in vivo human CatB imaging by seeing if the vulnerable portion of a plaque, whose matrix is fragile enough to be easily separated from the plaque body and to be embolized to cause stroke in association with the procedure, would have strong proteolytic enzyme activity.
from each entire carotid endarterectomy specimen and subregions were calculated. The subregions included bulb, proximal portion of the proximal internal carotid artery (ICA), distal portion of the proximal ICA, common carotid artery, and external carotid artery (Figure 1A). To analyze the anatomic distribution of the protease-related signal, the mean CatB signal or mean MMP signal was mapped on a template (averaged accumulation map). Median NIRF signal intensities were calculated for CatB emboli images, too.

**Histology**

Each carotid specimen was examined grossly for macroscopic plaque ulceration and surface thrombus. Fresh-frozen microsections (5-μm thickness) were used for fluorescence microscopy imaging, hematoxylin and eosin staining, Masson-trichrome staining, and immunohistochemical staining (CatB, MMP-2, MMP-9, macrophages). Immunohistochemistry was performed using the avidin-biotin-peroxidase method. A vascular pathologist who was blinded to the clinical and imaging data examined all the gross/micropathy data. Semiquantitative pathological examination was performed to identify “definitely unstable plaques”15 (AHA grade VI16), which were defined as having rupture as well as thrombus, large hemorrhage, and thin inflamed cap.

**Results**

**NIRF CatB or MMP Imaging Senses the Activity of Inflammatory Proteases From Macrophages In or Around the Bulb and Complicated Areas of Human Carotid Plaques**

NIRF CatB or MMP imaging reflected the proteolytic enzyme activities from macrophages in complicated human atheromata (Figures 1 and 2). Ulcerated, hemorrhagic lesions were present at pathology in 18 of 41 endarterectomy specimens and showed strong signal in or around the ulcerations in 14 (77%) cases (11 of 13 lesions imaged with CatB, and 4 of 6 lesions imaged with MMP). Negligible NIRF signal was detected in the control imaging without the probes (Figure I).

In the mean CatB images or MMP images, strong protease-related signal was localized to the carotid bifurcation area (Figure 3). Quantitative data corroborated this (Table I). Briefly, strong CatB signal clusters (the highest or second-highest intensity lesions in the 5 subregions of each plaque) were observed mostly in the carotid bulb (18 of 23 cases) or in the proximal portion of the proximal ICA (19 of 23 cases). Likewise, most of strong MMP signal clusters were located in the bulb or the proximal portion of the proximal ICA (5 of 6 cases).

CatB activities did not differ between the carotid plaques from the patients with recent-onset ipsilateral embolic strokes within 1 month (n=11) and those with no such stroke in the past month (n=20; Mann–Whitney test; P=0.95). The mean CatB images of the 2 groups, however, revealed a qualitative difference (Figure 3). In the symptomatic plaques, a bigger and stronger signal was located in the bulb area, whereas the signals from asymptomatic ones were scattered over somewhat larger areas.

**NIRF CatB Imaging of Emboli Dislodged During Carotid Stenting Reveals Strong Protease-Related Signal Corresponding to Inflammatory Changes**

We performed CatB imaging of emboli that had been dislodged from the carotid atheromata during angioplasty and...
stenting procedure (Figure 4). All the emboli collected in the protection device had strong CatB-related activity relating to macrophages. The CatB activities tend to be higher in the cases complicated with stenting-associated acute embolic cerebral infarctions (n = 11; median = 66 arbitrary units [AU]) than in those without such lesions (n = 4; median = 53 AU) on diffusion-weighted MRI performed at 24 hours after the intervention. However, this association was not statistically significant (P = 0.09; Mann–Whitney test).

The Degree of Carotid Stenosis or Ultrasonographic Echolucency Is Weakly Correlated With the CatB NIRF Signal Intensity
CatB NIRF signal intensity correlated with the degree of carotid stenosis (Figure 5A), diameter reduction measured on longitudinal section images of the duplex ultrasonography (r = 0.51; P = 0.005). MMP imaging data showed a similar trend (Figure 5B). Ten of the 15 (67%) cases with 80% to 99% stenosis had CatB activities higher than the median value of 87.8 AU (Figure 5C). Only 5 of the 15 (33.3%) cases with <80% stenosis had CatB activities higher than the median activity. However, it should be noted that all but 2 cases (6/8) in the 70% to 79% range of stenosis, in which carotid endarterectomy is indicated according to the current practice guidelines, had CatB activities lower than the median value.

In the CatB cases, duplex ultrasonography and angiography showed that the most stenotic portions were in the distal portion of the proximal ICA (n = 12), proximal portion of the proximal ICA (n = 6), or carotid bulb (n = 5). In the MMP cases, the most stenotic portions were in the distal portion of the proximal ICA (n = 2), proximal portion of the proximal ICA (n = 3), or carotid bulb (n = 1). CatB signal intensity in the most stenotic portion of each plaque was strong in 14 of 23 cases. MMP signal intensity in the most stenotic portion of each plaque was strong in 4 of 6 cases. It is notable that not infrequently (9 of 23 in CatB cases and 2 of 6 MMP cases), the most stenotic portions had only weak signals.

Grayscale median values of echodensities measured from 65 regions of interest on the duplex ultrasonography tended to be inversely and weakly correlated with the median CatB signal intensities from corresponding areas on the molecular optical imaging (Figure 5D), which showed a marginal significance (r = 0.24; P = 0.06). In addition, echolucent or heterogeneous plaques were non-significantly associated with high protease activities (please see http://atvb.ahajournals.org for further details).

CatB NIRF Signal Intensity Is Lower in the Plaques From Patients With Statin Medication
CatB activities tended to be lower in the statin users than in the nonusers (median = 88.2 vs 93.3 AU; P = 0.12; Mann–Whitney test). When the CatB activities were dichotomized, the lower CatB group was more frequently associated with statin use (10/15) than the higher CatB group was (5/16; P = 0.049; χ² test; Table II).

Pathology-Proven Definitely Unstable Plaques Are Relatively Frequent in the Higher CatB Group Compared With the Lower CatB Group
Semiquantitative pathological examination could be performed in selected cases (22 of the 31 CatB plaque imaging) because tissue status of some carotid specimens was not optimal for histopathologic examinations, probably because of the long incubation time. When CatB activities were dichotomized based on the median value of the 22 cases (86.6 AU), in the higher CatB group 5 of 11 (45%) carotid plaques were classified as definitely unstable. In the lower CatB group, 1 out of 11 (9%) carotid plaques were classified as definitely unstable. Such an association was not observed when the cases were dichotomized based on the median echodensities (22.0 AU); there were 3
definitely unstable plaques (27%) in both the higher (n=11) and lower (n=11) echodensity groups.

**Discussion**

Conventional imaging approaches including angiography, ultrasound, and MRI/ MRA offer primarily structural information, whereas molecular imaging can provide underlying molecular information on pathological processes such as inflammatory protease activities. We present observational evidence that plaque vulnerability can be assessed by means of molecular imaging, which complements and adds to traditional anatomic information.

To investigate the relationship between structural and molecular information related to the carotid atheromata, we correlated protease-related signal levels with the degree of stenosis and ultrasonographic appearance. We also compared protease signal intensity profiles to the site of maximum anatomic stenosis. We showed that the carotid plaques from the patients with higher-degree stenosis were more inflammatory, as judged by higher CatB or MMP activity, than those with lower-degree stenosis were. However, the correlations were rather weak, and not infrequently the most stenotic portion of a plaque had relatively weak protease activity. Thus, there is correspondence but also some informative divergence between anatomic and molecular imaging. On ultrasound, vulnerable plaques are thought to be echolucent and heterogeneous. We showed that CatB protease activity tended to be stronger in the heterogeneous echolucent plaques; the correlation, however, was not strong. This again suggests that molecular imaging provides new data not accessed by anatomy-based imaging.

Histological composition of the carotid plaque has been associated with plaque instability and presenting vascular symptoms, influencing the prognosis and therefore the indication for carotid intervention. We demonstrated that the molecular optical imaging could sense atherosclerosis pathophysiology.
Geometrically, higher CatB or MMP-related signals were observed in the bifurcation area than in the straight portions, including the common carotid artery or distal part of the proximal ICA. Unlike straight arteries, the regions of stenosed or branched arteries being exposed to disturbed flow conditions are prone to atheromatous development with high protease activities. These proteases go on to fragment elastins and probably break-up the fibrous plaque. In our study, strong protease activity was frequently observed in or around the unstable ulcerated and hemorrhagic areas of the carotid atheroma. This is in agreement with a recent immunohistochemical study and an in situ hybridization study. However, it is important to note that currently available antibodies do not distinguish active proteases from inactive precursors that lack proteolytic capacity,
and thus our study based on the activatable NIRF probes is likely yielding information more applicable to the pathophysiology of plaque rupture.3

Previously, carotid endarterectomy specimens excised as intact cylinders were subjected to a standardized angioplasty procedure under radiological guidance in an ex vivo pulsatile flow model.24 Macrophage infiltration within the plaques correlated with emboli number and the plasma MMP-9 level.24 In the actual clinical setting, we demonstrated that all the emboli from carotid plaques, which were collected in each patient’s blood stream in vivo, had strong CatB-related imaging signal. These emboli are likely fragile portions of plaques, vulnerable to dislodgement and embolization during the catheterization, and having high proteolytic enzyme activity. We therefore believe that protease-sensing molecular imaging has potential to provide in vivo histopathology data reflecting the vulnerability of atherosclerotic plaques.

The ideal tool to identify vulnerable plaques would allow detection of lesions at high risk for vascular events and would allow the assessment of risk-altering treatments so that the success of therapy can be judged in a timely manner. In fact, pathology-proven definitely unstable plaques were more frequently found in the higher CatB group than in the lower CatB group. Moreover, therapeutic effect of statin could be reflected by the molecular optical imaging, although the CatB-lowering effect of statin was not accompanied by lower serum cholesterol levels.25 Contrary to our expectations, total CatB-related fluorescence did not differ in the plaques from the patients with recent-onset ipsilateral embolic strokes within 1 month and those without such a stroke. This might be partly explained by a selection bias; patients with definitely “stable” plaques, optimal negative controls, would have a relatively low chance of getting the surgery and being enrolled in the study. Nevertheless, CatB molecular imaging did show a difference in the distribution

Figure 5. Anatomic imaging data to correlate weakly with NIRF CatB or MMP imaging data. A and B, CatB activity of a plaque is higher when the carotid stenosis is more severe, for which diameter reduction on the duplex ultrasonography was used as the degree of stenosis (A). MMP imaging also showed similar relationship between the stenosis degree and the protease activity (B). C, Plaques having CatB activities higher than the median value (horizontal line) are relatively frequent in the group with 80% to 99% stenosis (grade 6) compared with the groups with <80% stenosis (grade 1–5). D, The CatB activities in 65 regions of interest from the plaques (n=21) tend to be lower when the echodensities of the corresponding areas on the duplex ultrasonography are higher.
pattern of the enzyme activity signal. In the symptomatic plaques, stronger signal was densely concentrated in the bulb area, whereas the signal from asymptomatic plaques was more diffuse. Hypothetically, if strong CatB activity is focally localized to a critical structure such as a thin fibrous cap overlying a necrotic core, total protease activity may not represent actual vulnerability of the plaque. In this context, molecular imaging and structural imaging might have to complement each other, rather than each serving as a stand-alone technique, for the identification of vulnerable plaques. In conclusion, our study suggests that future prospective clinical trials to evaluate the use of protease-based molecular imaging technologies in human atherosclerosis patients are warranted.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Detailed Methods

Protease-Activatable Probes

Cathepsin-B (CatB) activatable near-infrared fluorescent (NIRF) probes were purchased (ProSense 680, Visen medical, Woburn, MA; Company published information (www.visenmedical.com/products/fluorescence_agents) shows that the specificity of the probe is high for CatB, but small amounts of activation is also noted in other cathepsins. A polymeric nanoparticle-based matrix metalloproteinase-2/9 (MMP-2/9) activatable probe was synthesized as described previously\textsuperscript{1-3} with some modification. We created MMP-sensitive nanoparticles by conjugating Cy5.5-Glu-Leu-Pro-Gly-Arg-Gly-Lys(BHQ-3)-Gly-Gly-COOH, MMP-2/9 cleavable NIRF dye-peptide-quencher substrate, to chitosan-based polymeric nanoparticles (HGC). Based on the known MMP-2/9 substrate, PLGVRG,\textsuperscript{4} we designed an MMP activatable fluorogenic peptide using a combination of a linker peptide, and NIRF dye and quencher: Cy5.5 (excitation/emission, 675/690 nm) and black hole quencher-3 (BHQ-3, maximal absorption in the 620 to 730 nm).\textsuperscript{5} The fluorogenic peptide, Cy5.5-GPLGVRGK(BHQ-3)GG, was synthesized using standard solid-phase Fmoc peptide chemistry.\textsuperscript{3} We labeled the MMP activatable fluorogenic peptide on the surface of hydrophobic 5β-cholanic acid conjugated glycol chitosan nanoparticles.\textsuperscript{2} The resulting particles were spherical and approximately 250 nm in diameter. The imaging probes were well dispersed in the reaction buffer (100 mM Tris, 5 mM calcium chloride, 200 mM NaCl, 0.1% Brij, pH 7.5) and NIRF signals were completely quenched when visualized with a Kodak Image Station 4000MM equipped with a Cy5.5 filter system. Before and 24h after the incubation, molecular optical imaging was performed using a NIRF imaging machine with a charge-coupled device camera (CoolSnap EZ, Roper Scientific, Tucson, AZ). At first, we tested whether control imaging without the CatB or MMP-2/9 probes produced Cy5.5 NIRF signal or not.
In Vitro Characterization of the MMP-2/9 Probe. The MMP-2/9 selectivity of the imaging probe was examined in vitro by incubating the probe (100 pM) in a cuvette containing the reaction buffer and 15 nmol/L of activated MMP-2, MMP-3, MMP-7, MMP-9, and MMP-2 inhibitor (1 mmol/L, 1,10-phenanthroline). Inactive MMPs were activated by incubating them with 2.5 mmol/L of p-aminophenyl mercuric acid in the reaction buffer at 37°C for 60 min. After incubation in the reaction buffer at 37°C for 120 min with the respective enzymes and inhibitor, the NIRF emission signals of the samples were measured using a spectrofluorometer (Hitachi F-7000, Tokyo, Japan) with a fixed excitation wavelength of 675 nm.

In order to see if CatB could activate the MMP-2/9 probe or not, CatB (R&D Systems, Minneapolis, MN) was activated by incubating them with 25mM 2-(N-morpholino)ethanesulfonic acid (MES) and 5mM dithiothreitol, pH 5.0, at room temperature for 15 min before initiation of the assay. The spectrofluorometer was used to monitor fluorescence intensity while incubating the MMP-2/9 probe (23µg/ml) in the reaction buffer (25mM MES, pH 5.0) containing 15nM of activated CatB at room temperature.

In another experiment to see if MMP-related NIRF signal intensity is dependent on numbers of macrophages, murine macrophage Raw 264.7 cells in DMEM (Dulbecco’s modified Eagle’s medium, Gibco, NY) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100g/ml streptomycin, were seeded onto a 96 well plate to reach different confluencies (0, 7.5×10^2, 3×10^3, 1.2×10^4, 5×10^4 cells per well). On the next day, cells were washed once with PBS and further incubated in phenol red free / serum free medium containing the MMP-2/9 NIRF probe (final concentration 0.5M) for additional 7h. Cy5.5 NIRF images were acquired from a triplicate experiment using a NIRF imaging machine with a charge-coupled device camera (CoolSnap EZ, Roper Scientific, Tucson, AZ).

In Vivo Characterization of the MMP-2/9 Probe. We tested if the MMP-2/9 probe could sense the protease activity and reflect the pro-atherosclerotic effect of a western-type diet in mice. This supplementary animal study was performed in accordance with the NIH-Guide for the care and use of laboratory animals.
We used ApoE knock-out (ApoE−/−) mice (Japan SLC, Shizuoka, Japan) fed on a normal chow diet (n = 5, normal diet group) or western diet (0.2% cholesterol; n = 5, western diet group) for 10 weeks, which started 8 weeks after they were born. The animals were anesthetized with 2% isoflurane in a mixture of 30% oxygen and 70% nitrogen, and 3 nmol MMP-2/9 probe in 150µl DMEM was intravenously injected. Twenty four hours after the tail vein injection of the probe, the animals were euthanized, and the aortas were carefully excised for ex vivo NIRF reflectance imaging.

NIRF imaging and lesion quantification were performed as published before.6 The excised aortas were washed with DMEM three times and imaged ex vivo by using a NIRF imaging machine. White light and Cy5.5 NIRF images (1-sec acquisition) were acquired. After normalization, the median NIRF signal intensities of the entire tissues were measured using the histogram function of Adobe Photoshop CS3-Extended (Adobe Systems, San Jose, CA).

In another experiment to see the specificity of the MMP-2/9 probe at a tissue level, 8-week-old ApoE knock-out mice (n = 4) on a high cholesterol diet for additional 14 weeks were used. After the mice were anesthetized, aortas were excised, rinsed, and incubated in DMEM containing either 20M of MMP-2/9 inhibitor II (Calbiochem, San Diego, CA) or dimethylsulfoxide (DMSO) for 1h. Then, MMP-2/9 probe (final concentration 0.5M) was added to the medium, and aortas were further incubated for additional 24h. Cy5.5 NIRF images were acquired as described above.

**Duplex Ultrasonography.** Duplex ultrasonography was performed according to the standardized protocol as published before.7,8 Patients were examined in the supine position with the head slightly elevated and the neck extended. All images were obtained on HDI 3000 scanner (Advanced Technology Laboratories, Bothell, WA) or iU22 ultrasound system (Philips, Bothell, WA) using 5- to 10-MHz linear-array transducer. The examination produced real-time displays of high-resolution gray-scale images and simultaneous color-encoded blood flow information, for which all sonographic parameters were optimized. Longitudinal and transverse scans of the entire common carotid artery (CCA) were obtained from the clavicle to the carotid bifurcation. Then, longitudinal and transverse scans of the internal and external carotid arteries were also obtained from the carotid bifurcation to
their disappearance beyond the angle of the mandible. Sonographic data of representative segments and any suspicious pathology were stored for later inspection and reevaluation.

**Sonographic Plaque Characterization.** First, plaque stenosis estimates (percentage reduction of diameter) were obtained from the gray-scale sonographic images. In addition, based on the plaque estimate and flow velocities—peak systolic and end-diastolic flow velocities of the internal carotid artery (ICA) and ICA/CCA peak systolic velocity ratio—the degree of carotid stenosis was classified as normal, <50%, 50–69%, 70% and higher stenosis, or near occlusion. Second, computer-assisted measurement of overall plaque echodensity was performed, in which the median of the frequency distribution of gray values of the pixels within the plaque (gray scale median or GSM) was measured. Adobe Photoshop with the histogram facility and ‘Curves’ option was used to normalize images, after which two reference points—blood and adventitia—were set to have GSM as 0–5 and 185–195, respectively. Echodensities of regions of interests on sonographic images were also acquired to correlate them with protease activities of corresponding areas on CatB or MMP images. Lastly, carotid plaque typing was performed, based on ultrasonic heterogeneity and/or echolucency.

Plaque morphology was classified as homogenous (p1) or heterogeneous (p2) by visual inspection of the image data. Then, computer-assisted characterization of plaque texture features was performed to redefine the Geroulakos classification, which was based on the percentage of the pixels having a gray scale greater than 25: plaque types 1 (less than 15%, hypoechoic), 2 (15–50%, mainly hypoechoic), 3 (51–85%, mainly hyperechoic), 4 (more than 85%, hyperechoic), 5 (plaques with extensive calcification and acoustic shadowing that cannot be assessed).

**Stroke Mechanism Analysis.** Mechanism of cerebral infarction was determined as previously described, focused on delineating embolism that had originated from the stenotic extracranial carotid artery, for which we used a published algorithm based on magnetic resonance (MR) angiography findings and ischemic lesion pattern on diffusion-weighted MR images. Plaques that had caused a recent ipsilateral embolic stroke within one month were classified as symptomatic plaques.
**NIRF Imaging and Lesion Assessment.** NIRF reflectance imaging and lesion quantification were performed as published before with some modification. Briefly, the carotid specimens were opened up to allow imaging of the luminal surface. White light and Cy5.5 NIRF (100-ms acquisition) images were acquired. Before the first Cy5.5 image of a tissue was acquired, NIRF imaging (100-ms acquisition) of a reference standard (30µmol/L free Cy5.5 dye) was performed. Adobe Photoshop was used to normalize the image by dragging the left lower point of the ‘Curves’ to the right to increase the ‘Input’ value (from 0) and stiffness of the originally 45° linear-slope, after which two reference points—the standard and background—were set to have mean gray-scale pixel intensities as 20 and 0, respectively. The same adjustment was applied to the tissue image. In most of the cases, the applied ‘Input’ values were 100–120. After the normalization, median NIRF signal intensities (gray-scale pixel intensities from 0 to 255) from each entire carotid endarterectomy specimen and sub-regions. To analyze the anatomical distribution of the protease-related signal, the mean CatB signal or mean MMP signal was mapped on a template. The mean CatB or MMP images, averaged accumulation maps from each group of source images after being transformed to fit into the template with the pre-defined sub-regions, was acquired using custom-built software DUIH_Image. The transformation was performed using the transform tool and warping tool of Adobe Photoshop (please see the transformation.mpg file). Median NIRF signal intensities were calculated for CatB emboli images, too. Likewise, quantification of the NIRF imaging data from the supplemental experiments was performed.

**Histology.** Each carotid specimen was examined grossly for macroscopic plaque ulceration and surface thrombus. Then, it was divided into 5–7 pieces according to the pre-defined 5 subregions. When the subregion CCA or distal proximal ICA was relatively long for one piece, it was divided into two pieces. Every fragment was transected serially (5mm thickness) and frozen in OCT compound. A section showing maximum thickness in each fragment was selected to get five adjacent fresh-frozen micro-sections (5µm thickness) for H&E staining, Masson-trichrome staining, and immunohistochemical staining (CatB, MMP-2, MMP-9, macrophages). Immunohistochemistry was
performed using the avidin-biotin-peroxidase method (see the below). A vascular pathologist who was blinded to the clinical and imaging data examined all the gross-/micro-pathology data. Semi-quantitative pathologic examination was performed to identify definitely unstable plaques\(^{15}\) (≈ AHA grade VI\(^{16}\)), which were defined as having rupture, thrombus, large hemorrhage, and thin inflamed cap. The other plaques were ones with various degrees of inflammation and hemorrhage, but without rupture.

A fluorescence microscopy (IX81-ZDC, Olympus, Tokyo, Japan) imaging was used to visualize the distribution of Cy5.5 fluorescence (5-sec acquisition) in plaque cryosections. Nearby sections were used for histology as described below.

Sections treated with 0.3% of hydrogen peroxide were incubated for 60 min with primary antibodies, followed by biotinylated secondary antibodies (EnVision, DAKO, Denmark). Macrophages were identified with anti-CD68 antibody (1:100, DAKO, Denmark). Protease activities were identified with rabbit polyclonal CatB antibody (1:75, Biovisions, Mountain View, CA) or MMP-2 / 9 antibodies (1:200 / 1:500, Chemicon, Temecula, CA). The reaction was visualized with DAB substrate and counterstained with Harris hematoxylin solution. Tissue sections were viewed and digitally captured using a Nikon Eclipse 800 microscope (Nikon, Japan) and compiled using Adobe Photoshop.

**Statistical Analyses.** Data are presented as mean ± SD. The Mann-Whitney test was used for comparison of continuous variables between groups. Chi-square test or Fisher’s exact test was used to compare proportions between groups. Bivariate correlations were calculated to see how two variables are related. A value of \(P<0.05\) was considered statistically significant.
Supplemental Results

Control Imaging

As shown in the representative case (Figure 1), negligible NIRF signal was detected in the plaque incubated with DMEM without the CatB or MMP-2/9 probes.

The In Vitro Study Demonstrated that the MMP Probe Could Be Used for Quantitative Measurement of MMP-2/9 Activity. Spectrofluormetry showed that significant recovery of the NIRF signal occurred against MMP-2 and MMP-9 (approximately 25 and 15-folds for MMP-2 and 9 vs. without MMP) and the fluorescence signal intensity was decreased in the presence of the MMP-2 inhibitor (Figure 2A). In addition, the probe showed the proportional relationship between recovered fluorescence signals and different MMP-2 concentrations (0.75, 1.9, 3.8, 7.5, and 15 nmol/L) ($r^2 = 0.99$, $P = 0.00$, Pearson correlation) (Figure 2B). Moreover, as shown in the Figure 3, the MMP probe could not be activated by CatB. We also showed that MMP-related NIRF signal was dependent on numbers of macrophages (Figure 4).

The In Vivo Study Demonstrated that the MMP Probe Could Be Used for Quantitative Imaging of MMP-2/9 Activity in Mice Atheromata. NIRF imaging with the MMP-2/9 probe visualized the enzymatic activities in the atheromata of ApoE knock-out mice. Atherogenic effect of the western diet was demonstrated by the MMP-2/9 NIRF imaging. As shown in the Figure 5, the MMP-2/9-related signal intensities were significantly higher in the western diet group (111.6 ± 87.2) than in the normal diet group (9.4 ± 11.8, $P < 0.05$, Mann-Whitney test). In addition, Figure 6 shows that the MMP-2/9 inhibitor could reduce the generation of the MMP-2/9-related NIRF signal from the mice atheromata.

Echolucent or Heterogeneous Plaques Were Non-Significantly Associated with Low Protease Activities. When plaque types were classified as having homogenous or heterogeneous echodensity
by visual inspection, more heterogenous plaques tended to have CatB activities higher than the median value 87.8A.U. (Figure 7A), which however did not reach a statistical significance ($P = 0.10$, Chi-Square test). In the MMP imaging ($n = 6$), three of four heterogeneous plaques had MMP activities higher than those of the other two homogenous plaques. When the CatB signal intensities were plotted against the five sonographic plaque types that were classified using the computer-assisted characterization method, about half (8 of 18) the echolucent plaques (type 1 and 2) had CatB activities higher than the median value. In cases of echogenic plaques, relatively less (3 of 9) had CatB activities higher than the median value (Figure 7B).

**CatB or MMP-2/9 immunoreactivities co-localize with CD-68 positive macrophages.**

As shown in the Figure 8, the locations with CatB or MMP immunoreactivities overlap with the areas infiltrated with CD-68 positive macrophages.
Supplemental References


Figure 1. Near-infrared fluorescent (NIFR) imaging without the CatB or MMP-2/9 probes shows that the intensity of NIRF signal from the plaque (arrow) was similar to that from the surrounding background.
Figure 2. Specificity of the MMP-2/9 imaging probe. Fluorescence emission at 690 nm in the presence of various enzymes after 120 min incubation (A). Fluorescence emission at 690 nm in the presence of various concentrations of MMP-2 after 120 min incubation (B). A.U. = arbitrary units
Figure 3. The MMP-2/9 probe can be activated by MMP-2 (black dots) but not by CatB (white dots).
Figure 4. MMP-2/9 near-infrared fluorescent (NIRF) signal intensity depends on numbers of macrophages (Raw 264.7 cells). When no cells are present, Cy5.5 NIRF signal is not observed (A). MMP-related NIRF signal intensity increases (B–E) as the cell confluency increases (b–e). Quantified data are the mean ± SD (arbitrary units, A.U.) of a triplicate experiment.
Figure 5. Quantification of MMP-2/9 near-infrared fluorescent (NIRF) imaging signal intensity in mice on a normal diet (n = 5) or western diet (n = 5). The MMP-related signals (arbitrary units, A.U.) from the atheromatous aortas are significantly higher in the western diet group than in the normal diet group. Inlet = representative light and NIRF images of each group.
Figure 6. MMP-2/9 near-infrared fluorescent (NIRF) imaging signal can be suppressed by the use of an MMP-2/9 inhibitor. In the representative NIRF imaging of tissue-cultured aortas after being excised from the ApoE knock-out mice (left two images), co-incubation of the MMP-2/9 inhibitor (right two images) prevented the development of the MMP-related Cy5.5 signal.
Figure 7. Plaques having CatB activities higher than the median value (horizontal line) are relatively frequent in the plaques with heterogeneous echodensities (P2, A) or low echodensities (plaque type 1 and 2, B) compared with the others (See the Methods for the plaque classifications).
Figure 8. Areas with positive CatB or MMP-2/9 immunoreactivity overlap with areas infiltrated with CD-68 positive macrophages in human carotid atheromata. Scale bar = 200µm.
Supplemental Tables

Supplemental Table I. Distribution of Strong Protease Activity Signals in the Carotid Plaques

<table>
<thead>
<tr>
<th>Signal</th>
<th>Intensity</th>
<th>Location</th>
<th>Bulb</th>
<th>p-pICA</th>
<th>d-pICA</th>
<th>CCA</th>
<th>ECA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CatB</td>
<td>Strong</td>
<td></td>
<td>18</td>
<td>19</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td></td>
<td>5</td>
<td>4</td>
<td>11</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>23</td>
<td>23</td>
<td>15</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>MMP</td>
<td>Strong</td>
<td></td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

p-pICA=proximal portion of the proximal internal carotid artery, d-pICA=distal portion of the proximal internal carotid artery, CCA=common carotid artery, ECA=external carotid artery. For definition of strong or weak signal intensity, please see the Methods.
Supplemental Table II. Clinical Features of Lower CatB vs. Higher CatB Cases of Plaque

Imaging

<table>
<thead>
<tr>
<th></th>
<th>Lower CatB (n=15)</th>
<th>Higher CatB (n=16)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median CatB Activity (arbitrary unit)</td>
<td>79</td>
<td>96</td>
<td>0.00</td>
</tr>
<tr>
<td>Median Degree of Carotid Stenosis (%)</td>
<td>77</td>
<td>77</td>
<td>0.66</td>
</tr>
<tr>
<td>Median Age (years)</td>
<td>70</td>
<td>66</td>
<td>0.17</td>
</tr>
<tr>
<td>Male</td>
<td>11 (73%)</td>
<td>13 (87%)</td>
<td>0.60</td>
</tr>
<tr>
<td>Smoking</td>
<td>6 (40%)</td>
<td>10 (67%)</td>
<td>0.21</td>
</tr>
<tr>
<td>Hypertension</td>
<td>12 (80%)</td>
<td>15 (94%)</td>
<td>0.33</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>7 (47%)</td>
<td>9 (56%)</td>
<td>0.72</td>
</tr>
<tr>
<td>History of Angina or Myocardial Infarction</td>
<td>2 (13%)</td>
<td>5 (31%)</td>
<td>0.39</td>
</tr>
<tr>
<td>Any Ipsilateral Cerebral Infarction</td>
<td>13 (87%)</td>
<td>10 (63%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Recent-onset (≤ 1 month) Ipsilateral Embolic Cerebral Infarction</td>
<td>6 (40%)</td>
<td>5 (33%)</td>
<td>0.61</td>
</tr>
<tr>
<td>Antihypertensive Treatment</td>
<td>12 (80%)</td>
<td>15 (94%)</td>
<td>0.33</td>
</tr>
<tr>
<td>Antiplatelet Treatment</td>
<td>12 (80%)</td>
<td>13 (81%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Statin Treatment</td>
<td>10 (67%)</td>
<td>5 (31%)</td>
<td>0.049</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>152</td>
<td>161</td>
<td>0.81</td>
</tr>
<tr>
<td>Low Density Lipoprotein Cholesterol</td>
<td>104</td>
<td>108</td>
<td>0.90</td>
</tr>
<tr>
<td>High Density Lipoprotein Cholesterol</td>
<td>39</td>
<td>46</td>
<td>0.46</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>128</td>
<td>128</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Two patients underwent bilateral surgeries. Both carotid specimens of one patient belonged to the lower CatB group. In the other patient, one specimen belonged to the lower CatB group and the other specimen belonged to the higher CatB group. Mann-Whitney test was used for comparison of continuous variables between the groups. Chi-square test or Fisher’s exact test was used to compare proportions between the groups.
**Supplemental Table III. Medication and Other Major Comorbid Diseases**

<table>
<thead>
<tr>
<th></th>
<th>CatB Plaque (n=31)</th>
<th>CatB Emboli (n=15)</th>
<th>MMP (n=6)</th>
<th>Control (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other Major Comorbid Diseases</td>
<td>3 (10%)</td>
<td>3 (20%)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>HTH</td>
<td>Esoph. Ca.</td>
<td>ASO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCC</td>
<td>Nasal Ca.</td>
<td>ASO</td>
<td>ASO</td>
</tr>
<tr>
<td>Antihypertensive Treatment</td>
<td>27 (87%)</td>
<td>12 (80%)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>CCB</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCB + ARB</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ARB</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CCB + BB</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Other Drugs or Combinations</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Antiplatelet Treatment</td>
<td>25 (81%)</td>
<td>15 (100%)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Aspirin</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Aspirin + Clopidogrel</td>
<td>8</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin + Cilostazol</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cilostazol</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other Drugs or Combinations</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Four patients underwent bilateral carotid surgeries. Bilateral carotid specimens of two patients were used for CatB (cathepsin-B) plaque imaging. Each of bilateral carotid specimens from two patients was used for CatB (n=2), MMP (matrix metalloproteinase-2/9, n=1), control (n=1) plaque imaging, respectively. HTH = hypothyroidism, HCC = hepatocellular carcinoma, ASO = atherosclerosis obliterans, Esoph. Ca. = esophageal cancer, CCB = calcium channel blocker, ARB = angiotensin receptor blocker, BB = beta blocker.