Characterizing Cathepsin Activity and Macrophage Subtypes in Excised Human Carotid Plaques

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Background and Purpose—Atherosclerosis is a leading cause of mortality worldwide, contributing to both strokes and heart attacks. Macrophages are key players in atherogenesis, promoting vascular inflammation and arterial remodeling through cysteine cathepsin proteases. We used a cathepsin-targeted activity-based probe in human carotid plaque to assess its diagnostic potential and evaluate macrophage subtypes ex vivo.

Methods—Carotid plaque specimens surgically removed during endarterectomy from 62 patients (age range, 38% female, 28% symptomatic) were graded pathologically as either stable (Grade 1) or unstable (Grade 2 or 3). A cathepsin activity-based probe was used to quantify individual cathepsins in plaque tissue and macrophage subtypes.

Results—Cathepsin B and S activities were increased in unstable carotid plaques. They were quantified using the probe to biochemically investigate individual cathepsins (Cathepsin B and S: 0.97 and 0.90 for grade 3 versus 0.51 and 0.59 for grade 1; \(P=0.006\) and \(P=0.03\) arbitrary units (AU), respectively). Higher cathepsin activity was observed in carotid plaques from symptomatic patients (Cathepsin B and S: 0.65 and 0.77 for asymptomatic, 0.99 and 1.17 for symptomatic; \(P=0.008\) and \(P=0.005\) AU, respectively). Additionally, it was demonstrated that M2 macrophages from unstable plaques express cathepsin activity 5-fold higher than M2 macrophages from stable plaques (25.52 versus 5.22; \(P=0.008\) AU).

Conclusions—Targeting cathepsin activity in human carotid plaques may present a novel diagnostic tool for characterizing high-risk plaques. Novel cathepsin activity patterns within plaques and macrophage subpopulations suggest their involvement in the transition to active disease. (Stroke. 2016;47:1101-1108. DOI: 10.1161/STROKEAHA.115.011573.)

Key Words: activity-based probe ■ atherosclerosis ■ cathepsins ■ macrophages ■ optical imaging

The formation and progression of atherosclerotic plaques is characterized by accumulation of oxidized lipids and inflammatory cells covered by a fibrous cap of smooth muscle cells and collagen-rich matrix. Most severe clinical events result from plaque rupture, which exposes prothrombotic material in the plaque to the blood and causes intraluminal thrombus formation. Plaques vulnerable to rupture are characterized by a thin fibrous cap, a large lipid/necrotic core, positive vascular remodeling, and substantial infiltration of inflammatory cells. In particular, macrophages have emerged as a key cellular element of atherosclerotic plaque pathogenesis and pose a significant risk for causing plaque rupture. Recently, studies have observed heterogeneous subgroups of macrophages, such as M1 and M2, in human atherosclerotic lesions.

Progressive necrosis of foam cells causes secretion of several factors that sustain the inflammatory processes and produce tissue-degrading proteases, such as matrix metalloproteinases and elastases. Plaque destabilization is attributed mostly to extracellular matrix (ECM) degradation (collagenolysis elastolysis) by multiple ECM degrading enzymes, such as matrix metalloproteinases, serine hydrolyses, and cysteine proteases. Cysteine proteases, including cathepsins B, L, and S, display profound ECM catabolic activity in vitro and were found to be overexpressed in activated macrophages and advanced atherosclerotic lesions. Cathepsin B, L, and S play key roles in macrophage function, with their mRNA expression and activity increasing significantly in the M2 phenotype. Additionally, M1 macrophages show elevated cathepsin S and L mRNA levels. Moreover, both mRNA and protein levels of cathepsin B, L, and S are overexpressed in murine atherosclerotic plaques. Similarly, in advanced human atherosclerotic plaques, cathepsin B and S were found overexpressed within macrophages.
endothelial cells, and fibrous cap smooth muscle cells; they were shown to be sensitive to proinflammatory stimuli.\textsuperscript{9,10} In normal human arteries, however, cathepsin B and S protein are poorly expressed.\textsuperscript{11,12} Furthermore, the cathepsin activity was shown to be involved in inflammation in IL1β processed and to contribute to the degradation of the ECM in the fibrous cap and destabilization of the plaque.\textsuperscript{11,13–15} Therefore, these proteases serve as markers for plaque inflammation and vulnerability.

Given the previously identified role of cathepsins in plaque progression, compounds that can report on cathepsin activity in atherosclerotic plaques should allow studies of their involvement in plaque progression and distinguish between vulnerable and stable plaques.

We hypothesize that a fluorescent cathepsin activity-based probe (ABP), GB123, which we have previously developed,\textsuperscript{16,17} may enable identification of individual cathepsin activity in unstable plaques and the contribution of different macrophage subpopulations to this pathological state. ABPs are small molecules that covalently bind to a target enzyme in an activity-dependent manner. Because ABPs covalently bind to their enzyme targets, they remain at the active site, allowing biochemical analysis of the target enzymes.

Methods

Detailed methods are described in methods section in the online-only Data Supplement.

Patient Samples

Carotid plaque specimens were collected from patients who underwent carotid endarterectomy after providing informed consent. The carotid endarterectomy specimens were collected from a total of 62 patients classified as with or without a history of cerebrovascular symptoms; details in SI in the online-only Data Supplement.

Pathological Plaque Classification

Human carotid plaque samples were collected immediately after endarterectomy. Samples were divided into 3 parts that were flash frozen in liquid nitrogen, cryosectioned, and histopathologically analyzed.

All plaques were classified into 3 groups on the basis of their morphology as described in Li et al.\textsuperscript{18} Grade 1 to 3 plaques were defined by fibrous cap thickness and integrity, lipid pool, leukocyte infiltration, internal elastic lamina, necrotic core, cholesterol crystal clefts, internal plaque hemorrhage, thrombosis, and neovascularization. Plaques were referred to as stable if graded 1, vulnerable if graded 2, and ruptured if graded 3. Unstable plaques were defined as Grades 2 and 3; details in SI in the online-only Data Supplement.

Biochemical Analysis of Cathepsin Activity in Human Carotid Lysates

Frozen tissue sample lysates (n=25) were treated with cathepsin B, L, and S ABP, GB123\textsuperscript{16} (probe structure in Figure 1 in the online-only Data Supplement), and inhibitor controls were run. SDS-PAGE was fluorescently scanned; details in SIII in the online-only Data Supplement.

Measuring Cathepsin Activity in Carotid Plaques

Fresh carotid specimens were washed, frozen in optimal cutting temperature compound (OCT), and cryosectioned. Serial sections of tissue samples were washed and treated with GB123 for 1 hour; inhibitor controls were applied. Samples were fixed, washed, and analyzed by fluorescence confocal microscopy; details in SIV in the online-only Data Supplement.

Ex Vivo Labeling of Plaque Samples With Fluorescent ABPs

Freshly resected carotid samples (n=19) were incubated with GB123 and then washed for 4 or 24 hours. Cathepsin inhibitor controls were applied. Samples were imaged using an IVIS Kinetic. Plaque serial cryosections stained with cathepsin B, cathepsin S, and CD68 antibodies were analyzed by fluorescent microscopy; details in SV and SVI in the online-only Data Supplement.

Isolation of Cells From Carotid Plaque for Fluorescence-Activated Cell Sorter Analysis

Freshly resected human carotid samples (n=12) were incubated with GB123 for 1 hour and then embedded in OCT. After collecting stained plaques, the OCT-embedded specimens were thawed and washed. Single cell suspension was analyzed by fluorescent-activated cell sorter using CD45, CD86, or CD206 (mannose receptor) antibodies and GB123 labeling; details in SVII in the online-only Data Supplement.

Statistical Analysis

Data were expressed as means±SD values. Statistical analyses were done in Graphpad prism 6. Kolmogorov–Smirnov and Shapiro–Wilk tests were used to determine whether the data follows the normal distribution, and comparisons were done using 2-tailed Student’s t test and 1-way analysis of variance with false discovery rate correction set to 0.05 and Dunnett’s test, respectively, to account for multiple comparisons. Receiver–operator characteristic curve computed nonparametrically with 95% confidence intervals using the null hypothesis that the area under the curve equals 0.50. All experiments were performed in a double-blind fashion.

Results

Cathepsin Activity in Human Atherosclerotic Plaques

Plaques were collected from carotid endarterectomy patients between the ages of 57 and 87 years, 38% female, and 28% symptomatic. A representative computed tomographic angiogram before surgery is shown in Figure IIA in the online-only Data Supplement. Carotid plaques were divided into 3 stages according to their morphological alterations\textsuperscript{18} as described in the Methods section, with representative images shown in Figure IIB in the online-only Data Supplement. Carotid plaques were divided into 3 stages according to their morphological alterations\textsuperscript{18} as described in the Methods section, with representative images shown in Figure IIB in the online-only Data Supplement. ABP GB123\textsuperscript{16}, which labels active cathepsin B, L, and S enzymes, was applied to measure cathepsin activity in the different types of carotid plaques (n=25; for probe structures, see Figure I in the online-only Data Supplement). Importantly, using the novel fluorescently labeled cathepsin ABP conjugates allowed us to perform a specific biochemical analysis of the activity of individual cathepsins by running SDS-PAGE of tissue lysates. A representative fluorescent scan of a gel containing lysates of various lesion grades is shown in Figure 1A. The intensity of the bands at 31 and 26 kDa corresponds to the molecular weights of cathepsins B and S, respectively, as confirmed by immuno-precipitation (Figure III in the online-only Data Supplement). Importantly, it was found that the surrounding tissue near the plaque had lower cathepsin activity than the plaques themselves in almost all cases of Grade 2 and 3 (Figure 1A). Cathepsin L activity was found in low levels in lysates of excised plaques,
probably because of the labeling of lysates in which cathepsin L activity is often damaged by the lysing process itself. It was, therefore, decided to pursue with cathepsin B and S only.

Quantification of the cathepsin B and S activities, as measured by the gel analysis, showed good correlation of cathepsin activity with plaque severity (Figure 1B). For cathepsin B, activity increased by ≈2-fold in unstable Grade 2 and 3 plaques in comparison to stable Grade 1 plaques: 0.90 and 0.97 versus 0.51, respectively, AU (P<0.02). For cathepsin S, activity increased close to 1.5-fold, but only in Grade 3 plaques: 0.59 versus 0.90 AU (P=0.03; Figure 1B). These findings were further corroborated by Western blot analysis of cathepsin protein expression in the human carotid samples (Figure IV in the online-only Data Supplement). Using the GB123 probe, cathepsin B and S activity was compared in carotid plaques from symptomatic (n=7) and asymptomatic patients (n=18). When cathepsin B and S activities were quantified, it was found that carotid plaques from symptomatic patients displayed significantly higher cathepsin activity (≈1.5 fold) compared with plaques from asymptomatic patients (cathepsin B: 0.65 versus 0.99 AU, P=0.008, and cathepsin S: 0.77 versus 1.17 AU, P=0.005; Figure 1C). Using receiver-operator characteristic curves, further testing was performed to assay whether cathepsin B and S activity could differentiate between symptomatic and asymptomatic patients. The area under the curve was found to be 0.84 and 0.88 for cathepsin B and cathepsin S, respectively, thus indicating the ability of GB123 to discriminate symptomatic patients (Figure 1D).

Cathepsin Protein Expression Correlates With Human Carotid Plaque Grade

The magnitude of cathepsins B and S protein expression was measured in serial sections (n=21) of human carotid plaque specimens. Similar to the activity, differential expression patterns between cathepsin B and cathepsin S were found. Although cathepsin B increased in both Grade 2 and Grade 3 plaques, cathepsin S only increased in Grade 3 plaques. As expected, expression of both cathepsins B and S was found to colocalize with CD68-positive macrophages, in both Grade 2 and 3 plaques (Figure IIC in the online-only Data Supplement). To further validate the findings, gene expression profiling, GEO (GSE41571), of macrophages obtained from stable or...
ruptured human carotid plaques was performed. Significant enrichment was found in genes involved in lysosomal activity, including cathepsins B and S in ruptured versus stable plaques (Figure VI in the online-only Data Supplement). Taken together, these data demonstrate that expression and activity of cathepsins B and S are progressive with atherosclerotic plaque severity.

**Cathepsin ABP for Ex Vivo Plaque Imaging**

To establish our ABP as an accurate diagnostic imaging tool for atherosclerosis, fresh dissected human carotid samples (n=19) were cut into pieces (Figure 2A, left and right), then incubated with GB123 applying inhibitor controls (Figure 2B). As the GB123 probe is constitutively fluorescent, even when unbound, no significant difference in fluorescent signals was detected between plaques pretreated with or without an inhibitor in the 4-hour washout time. However, after 24 hours of washout, a significant difference in probe retention was observed, suggesting that extended washing is required to remove the unbound, nonquenched probe. Specifically, there was an increase in the Cy5 signal relative to inhibited samples in unstable plaques (Grade 2 and 3 combined; n=12) compared with stable plaques (Grade 1; n=7; 1.95 versus 1.19 AU; P=0.008; Figure 2B and 2C).

**Cathepsin Activity Localizes to Macrophages in Human Carotid Plaques**

Although our quantitative analyses established a strong correlation between cathepsins B and S activities and vascular pathology, their potential cell source remains to be identified. Using confocal microscopy, it was shown that macrophages were responsible for increased cathepsin activity. First, it was determined that GB123 renders a reliable estimate of cathepsin activity by treating tissue sections (n=12) with the probe in the presence or absence of a selective inhibitor (Figure 3A). A clear and specific signal was detected in frozen sections, and inhibitor pretreatment almost completely abrogated the GB123 fluorescent signal. Then, to investigate probe penetration, whole tissue samples were treated with GB123, washed for 24 hours, after which fresh frozen OCT samples were prepared and sectioned. Here, the fluorescent images similarly demonstrated a strong Cy5 signal within the plaque (Figure 3B). In addition, the immunoreactive CD68, cathepsins B, and cathepsin S colocalized with the majority of the Cy5 signal, showing that the GB123 signal reflects macrophage-derived cathepsin activity and also that local macrophages exhibit strong cathepsin activity in human carotid plaques (Figure 3C).

Plaque macrophages exhibited 2 distinct phenotypes commonly referred to as M1 (proinflammatory) or M2 (anti-inflammatory). Although both cell types contribute to ECM remodeling, their relative contribution to atherogenesis in human subjects is incompletely understood. For this reason, it was decided to explore the phenotype of macrophages with high cathepsin activity using our novel GB123 ABP. To understand whether the macrophages within the plaques were principally M1- or M2-polarized macrophages, a phenotypic characterization of the relative abundance of macrophage subtypes in atherosclerotic plaques was performed. Tissue samples treated with GB123 were analyzed for the costimulatory molecule CD86 as a marker for M1 macrophages and the mannose receptor CD206 (MR) as a marker for M2 macrophages. Interestingly, from the CD45+ gated monocyte population, the CD86+ cells were found in similar percentages in both stable and unstable plaques, 86±4% and 88±2% respectively. In contrast, an increase in MR+ macrophages (so-called M2) was found in unstable plaques (46±1.5% versus 39±3%; P=0.017; Figure 4A). Most dramatically, when cathepsin activity in the CD86+ and MR+ cells isolated from the human carotid plaques were measured, there was a significant increase in MR+ cells labeled with GB123 observed in unstable compared with stable plaques (25±4% versus 5.0±0.8%; P=0.008). However, there was almost no difference in cathepsin activity of CD86+ cells in stable and unstable plaques (25±6% versus 28±6%, P>0.05; Figure 4B), indicating that a unique population of M2 macrophages may be responsible for the upregulation in cathepsin activity within the unstable plaque. Finally, flow cytometric analysis was used to compare the mean fluorescent intensity of Cy5 signal in CD86+ versus MR+ cell populations. Confirming our previous results,

**Figure 2.** Ex-vivo imaging with GB123. Freshly extracted human carotid plaques (n=19; A, left) were cut into pieces (A, right), then incubated with 0.2 μmol/L GB123 for 1 hour and washed with acetate buffer for 24 and 4 hours. In addition, control samples preincubated for 1 hour with GB111-NH2 inhibitor before GB123 were prepared. Samples were placed on a dark napkin and imaged with an IVIS Kinetic system using 640/695 excitation and emission filters. A nontreated carotid plaque was used as a negative control (not shown). A representative picture in (B), high cathepsin activity, in yellow, was found in probe-treated unstable plaques. C, For signal quantification, photon counts were obtained from the carotid plaques. The ratio of GB123 probe fluorescent signal vs probe signal with inhibitor pretreatment samples was calculated from stable and unstable plaques (Grade 2+3). In GB123-treated samples, a significant increase in Cy5 signal ratio between stable and unstable plaques was observed after 24 hours of plaques washing. Data are presented as mean±SD. Student’s t test with false discovery rate correction set to 0.05 to account for multiple comparisons was applied.
there was an increase observed in the ratio of Cy5 mean fluorescent intensity for MR+/CD86+ in unstable compared with stable plaques (1.6±0.1 versus 0.8±0.2 AU; \( P = 0.015 \); Figure 4C). Representative dot plots are showed in Figure VII in the online-only Data Supplement.

**Discussion**

The application of the small molecular cathepsin ABP GB123 for characterizing plaque stability has been demonstrated. A significant correlation between cathepsin activity and plaque grade was found, with the highest cathepsin activity observed in advanced plaques. Although cathepsin S expression was highly elevated only in ruptured Grade 3 plaques, cathepsin B was found to be highly elevated in unstable Grade 2 and 3 plaques relative to Grade 1, thus suggesting potentially different roles for these 2 cathepsins during plaque progression. Furthermore, there was a significant correlation between patients with symptoms and high cathepsin activity. The above results indicate that cathepsins B and S are involved in, and can be detected in, plaque progression and rupture and correlate with symptomatic events.

Cathepsin L was reported to be highly expressed in rupture-prone regions of atheromatous in 3 major cell types encompassing vascular lesions, such as endothelial cells, smooth muscle cells, and macrophages.\(^{19,20}\) In this study, however, cathepsin L activity was not reported because, initially, plaques were lysed, thus leading to a significant loss in cathepsin L activity (G. Blum and M. Bogyo, unpublished data, 2005). Thus, the significant contribution of cathepsin L in vascular injury should not be excluded.

Because macrophages play a key role in inflammatory cardiovascular diseases, there have been several classes of probes that have been described for imaging macrophages as a way to detect vulnerable plaques. These include iodinated, fluorescent, fluorinated, and magnetic nanoparticles (based on an iron-oxide core) that have been shown to accumulate in macrophages in plaques and in injured myocardium. These probes were shown to be useful as contrast reagents for optical, magnetic resonance imaging, positron emission tomography, or computed tomography modalities.\(^{2,5,21–29}\) Furthermore, macrophage plasticity is the focus of several recent studies.\(^{30}\) Different subsets of macrophages, such as M1 and M2, have...
been observed in human atherosclerotic lesions. Fluorescence-activated cell sorter analysis showed upregulation in levels of MR+ (M2) cells in unstable compared with stable plaques, whereas CD86+ (M1) macrophages were present in higher but similar quantities in stable and unstable plaques. By using the ABPs, it was shown that a specific phenotype of MR+ macrophages of unstable plaques have a 5-fold increase in cathepsin activity compared with stable plaque MR+ cells, whereas the cathepsin activity in CD86+ cells remained unchanged. This increase in MR+ cathepsin activity of unstable plaques suggests that the major source of elevated cathepsin activities in these plaques originates from MR+ cells, implying that they are involved in plaque rupture. In stable plaques, however, the cathepsin activity is most likely generated by CD86+ cells because they found in substantially higher levels than MR+ cells. Taken together, these data highlight the contribution of cathepsins B and S to the clinical sequelae of atherosclerosis and suggests that M2 macrophages play a key role in advanced vascular lesions.

Figure 4. Fluorescence-activated cell sorter (FACS) analysis of macrophages in stable and unstable plaques. Optimal cutting temperature compound–embedded human carotid tissue (n=12) treated with GB123, as described in Figure 4B, were thawed and washed with PBS. Tissues were treated with collagenase Type II, and a single cell suspension was analyzed by FACS for pan-leukocyte marker CD45+, CD86, and mannose receptor CD206 (MR) macrophages markers. A, Lesion macrophages isolated from stable and unstable plaques were gated on the pan-leukocyte marker CD45+ and analyzed by FACS to determine the expression CD86+ and MR+. No difference was found in the percentage of CD45+CD86+ macrophages in both stable (black bars) and unstable plaques (open bars). However, there is a small but significant increase in CD45+MR+ population in unstable plaques compared with stable plaques. B, The percentage of cells that were found triple positive for GB123 Cy5 signal and macrophages markers, CD45+CD86+ or CD45+MR+, were quantified from stable and unstable plaques (black bars) and (open bars), respectively. Although cathepsins activity of CD45+MR+ cells in unstable plaques increased significantly by 5-folds compared with stable ones, there was almost no change in cathepsins activity in CD45+CD86+cells from stable and unstable plaques. C, Flow cytometry histogram overlay of CD45+CD86+ and CD45+MR+ lesion macrophages isolated from stable and unstable plaques. Data represents the ratio of mean fluorescence intensity (MFI) of MR+ or CD86+ positive cells. A 2-fold increase in the ratio of Cy5 MFI of MR+ cells vs CD86+ cells were found in unstable compared with stable plaques. Data in (A) and (B) represent the means±SD of the frequency of positive cells.

The fluorescently labeled probe described here is unique because it enables ex vivo imaging of the vulnerable plaque with multiple utilities: The probe forms a covalent bond with its targets, therefore, allowing direct analysis of cathepsin activity in tissues using biochemical methods, fluorescent microscopy, and fluorescence-activated cell sorter. Thus, this probe can be used to analyze activity of multiple cathepsins simultaneously, as well as determine the location of these activities within the plaque tissue and the cell type expressing the cathepsin activity.

As the degree of stenosis provides an imperfect estimate to the risk of clinical events (eg, myocardial infarction, stroke), there is a need for methods that can distinguish between vulnerable and stable atherosclerotic plaques. GB123 has been used previously in vivo for noninvasive molecular imaging of cancer and has shown high potential to be applied for preclinical imaging of cathepsin activity in atherosclerotic mice models. Similarly, high molecular weight polymeric fluorescently quenched cathepsin substrate probes have been
previously described for cancer and atherosclerosis applications and found to be extremely useful.

With high molecular weight polymeric fluorescently quenched cathepsin substrate probes being translated to clinical use with the aid of endoscopic instruments, we consider fluorescent ABP methodology to be attractive for several reasons. First, our probe is a small molecule that can freely penetrate cells and, therefore, target a larger pool of active cellular enzymes, resulting in higher signals than high molecular weight substrate probes, as shown in cancer models. Second, the ABP allows for separation of individual cathepsin activities using biochemical methods. In addition, the ABP GB123 methodology can be translated to clinical use by changing the fluorescent tag with a 64Cu label that can be detected in greater depth with positron emission tomography imaging, as shown previously. Combining the potential clinical use of ABPs with present results correlating cathepsin activity with plaque grade and patient symptoms may allow accurate identification of individuals at risk for atherosclerosis-associated complications. The fluorescent cathepsin ABP described here enables assessment of vulnerable plaques and serves as an attractive tool for atherosclerosis research, with potential for both preclinical and clinical plaque imaging.

Conclusions

The molecular tools presented in this article provide significant advancements in atherosclerosis research, providing a novel diagnostic method and a novel activity pattern of cathepsin in the M2 macrophages of unstable plaques.

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Disclosures

Dr McConnell has the following competing interest: cardiovascular magnetic resonance imaging research grant from GE Healthcare and preclinical research grant from Tiara Pharmaceuticals. Dr McConnell is currently an employee of Verily Life Sciences (Mountain View, CA) on partial leave from Stanford University. Dr McConnell has the following competing interest: cardiovascular magnetic resonance imaging research grant from GE Healthcare and preclinical research grant from Tiara Pharmaceuticals. Dr McConnell has the following competing interest: cardiovascular magnetic resonance imaging research grant from GE Healthcare and preclinical research grant from Tiara Pharmaceuticals.

References


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Supplemental Material

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Supplementary Methods

SI. Patient samples
Carotid plaque specimens were collected from patients with 60-99% stenosis who underwent carotid endarterectomy at Hadassah - Hebrew University Medical Center. The study protocol was approved by the Institutional Review Board (Helsinki approval number HMO-09-0515). All patients underwent preoperative computed tomography (CT) scan. The degree of stenosis of the carotid arteries was determined by examination of carotid duplex ultrasounds and CTs. Based on the Washington criteria for the sonograms and the NASCET method for the CTAs, asymptomatic patients with stenosis greater than 80% and symptomatic patients with stenosis greater than 60% were selected for endarterectomy. The carotid endarterectomy specimens were collected from a total of 62 patients classified as with or without a history of cerebrovascular symptoms (i.e., amaurosis fugax, transient ischemic attack, or stroke).

SII. Pathological plaque classification
Human carotid plaque samples were collected immediately after endarterectomy and used in chronological order without selection. The first 25 plaques were used for immunohistochemistry and for biochemistry, the second group of 19 plaques was used for ex-vivo imaging, the 3rd group of 5 plaques was used for caspase 3 staining after inhibitor treatment. Most samples were divided into three parts; one part was flash frozen in liquid nitrogen, the second part was embedded and frozen in optimal cutting temperature tissue compound (OCT compound, Sakura, Tokyo, Japan), and the third part was fixed in 4% buffered formaldehyde solution and prepared for routine histopathological analysis following division into cross-sectional segments (rings) of approximately 5mm in thickness. The rings were embedded in paraffin; 5 μm sections were obtained, stained with hematoxylin and eosin, and examined using an Olympus BX51 microscope. We classified all plaques into three groups on the basis of their morphology1. Grade 1 plaques were defined as intact plaques with a fibrous cap >100 μm in thickness, no lipid pool formation, minimal leukocyte infiltration and intact internal elastic lamina; Grade 2 plaques were defined as intact plaques with lipid pool formation, infiltration of leukocytes and a fibrous cap <100 μm in thickness; and Grade 3 plaques were defined as ruptured plaques, often containing a large necrotic core, cholesterol crystal clefts, infiltration of leukocytes, internal plaque hemorrhage, thrombosis, neovascularization and a ruptured fibrous cap1. Plaques were referred to as “stable” if graded 1, “vulnerable” if graded 2, and “ruptured” if graded 3. “Unstable” plaques were defined as “vulnerable” plus “ruptured”, i.e., Grades 2 and 3.

SIII. Biochemical analysis of cathepsin activity in human carotid lysates
Frozen tissue samples (n=25) were lysed by three freeze-thaw cycles followed by dounce homogenization in cold lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mmol/L MgCl₂ and 50 mmol/L sodium acetate pH 5.5). For labeling studies 100μg protein in 30 μl of acetate buffer (50 mmol/L sodium acetate pH 5.5, 5 mmol/L MgCl₂, and 4 mmol/L DTT) was treated with 1 μmol/L GB123 for 30 minutes. As controls, indicated samples were pre-incubated for 20 minutes with 5 μmol/L of cathepsin inhibitor, GB111-NH₂, prior to GB123 labeling. The reaction was stopped by adding sample buffer (10% glycerol, 50 mmol/L Tris HCl, pH 6.8, 3% SDS and 5% β-mercaptoethanol, 0.1% bromophenol blue). Samples were boiled, and separated on a 12.5% SDS-PAGE, wet gels were scanned for fluorescence using a Typhoon Scanner (GE healthcare USA) at 635/670 nm. Band intensity was measured using ImageJ software and normalized to the intensity of the coomassie stained band.

SIV. Measuring cathepsins activity in carotid plaques
Supplemental Material

To examine cathepsins activity in histological sections, fresh carotid specimens were washed with normal saline and frozen in OCT compound for cryosection preparation and stored at ~80°C. Tissue samples were cut into 7µm thick sections using a CM 1900 cryotome. Serial sections of each tissue sample were collected and washed with acetate buffer (50 mmol/L sodium acetate pH 5.5, 5 mmol/L MgCl₂) and treated with 0.2 µmol/L GB123 for 1 hour. In addition, neighboring sections were pre-incubated for one hour with 10 µmol/L GB111-NH₂ as a control without specific cathepsin labeling. Slides were washed with PBS three times for 10 minutes. Tissue sample were fixed in 4% paraformaldehyde (15 min at 4°C) and washed 3 times with PBS 0.1% Tween for 10 minutes, then left in PBS overnight. The sections were analyzed for Cy5 fluorescence of GB123 with Olympus FV10i confocal microscope.

SV. Ex vivo labeling of plaque samples with fluorescent ABPs
Freshly resected human carotid samples (n=19) were washed with acetate buffer and incubated with 0.2 µmol/L GB123 for 1 hour (concentrations were tested between 0.1-2 µM and optimal concentration of signal over background was selected, data not shown), then washed with acetate buffer for 4 hours or 24 hours, respectively. In addition, part of the sample was pre-incubation for one hour with 10 µmol/L of the cathepsin inhibitor GB111-NH₂ as a negative control. Samples were imaged using an IVIS Kinetic system (PerkinElmer, USA) equipped with 640/695 excitation and emission filters. Twenty hours after probe washing, samples were incubated for 4 hours with 4% paraformaldehyde and with 30% sucrose overnight at 4°C. The samples were then embedded in OCT for cryosectioning. The tissue samples were cut into 7µm thick slices using a CM 1900 cryotome (Leica Microsystems, Wetzlar, Germany). Serial sections of each tissue sample were collected and stained with primary antibodies against human cathepsin B, cathepsin S and CD68 and then with a second fluorescent antibody coupled to Cy3. GB123 probe fluorescence was detected with a Cy5 filter and images were captured with an Olympus FV10i confocal microscope. Cathepsins activity in tissue was quantified by the ratio of Cy5 signal from ABP-treated tissue and the Cy5 signal from ABP pretreated with the cathepsin inhibitor.

SVI. Immunohistochemistry
To examine the location and expression of cathepsins, serial paraffin sections were exposed to the following primary antibodies: monoclonal mouse anti-human CD68 clone PG-M1 (1:100, DAKO, Denmark), rabbit anti-human cathepsin B (clone EPR4323) or cathepsin S (clone EPR5128) (1:50 Epitomics, Burlingame, CA, USA). The slides were reacted with peroxidase-conjugated anti mouse or rabbit IgG, and the brown signals were developed by immersing slides in the DAB (Dako). Controls were run without primary antibody. Sections were then counterstained with Gill's hematoxylin solution.

SVII. Isolation of cells from carotid plaque for FACS analysis
Freshly resected human carotid samples (n=12) were processed within 15 min of extraction. Tissue was washed with acetate buffer and incubated with 0.2 µmol/L GB123 for 1 hour. Tissues were washed in acetate buffer 24 hours at 4°C and fixed with 4% paraformaldehyde, kept in 30% sucrose overnight at 4°C, and then embedded in OCT. After collecting stained plaques, the OCT-embedded specimens were thawed and washed 3 times with PBS; then plaque tissue was minced into small pieces and placed into digestion medium consisting of DMEM supplemented with collagenase Type II (0.3%, Sigma–Aldrich). Tissues were rotated at 37 °C for 2 hours and then filtered through a 100µm filter to remove debris (Becton Dickinson, Franklin Lakes, NJ) then centrifuged at 300g at 4 °C for 8 min. Cells were washed with DMEM supplemented with 10% fetal calf serum, and with PBS three times. Cells were counted and 10X10⁶ cells were used for FACS experiments. Anti CD45-FITC (ABCAM) used as monocytes marker, ~7% of the cells were CD45 positive. Out of the CD45+ cells 10000 events were analyzed in each experiment. Lesional macrophages were co-stained with anti-CD45-FITC (ABCAM), CD86-PE (eBioscience, San Diego, CA, USA) or anti-CD206-PE (mannose receptor) (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Then, flow cytometry was performed to determine the expression of cell surface antigens and GB123 labeling using a LSRII flow cytometer (BD Biosciences). Data were analyzed with FACS express software (BD, BD Biosciences).
Supplementary Figure I. Cathepsin Probes and Inhibitor Structures

Structures of the inhibitor GB111-NH$_2$ and the non-quenched activity based probe GB123 labeled with Cy5, were published in $^3$ and $^2$ respectively.
Supplementary Figure II. Correlation between cathepsin protein levels and human carotid plaque grade

a) CT angiogram of carotid artery prior to endarterectomy. A representative image of a patient injected with iodine contrast agent and imaged with a CT scanner, picture is a single slice of the scan; arrow indicates location of narrowed artery and calcified lesion.

b) Pathological classification of human plaques (n=62) into three groups on the basis of their morphology. Grade 1 (stable) lesions are fibronodular. Grade 2 lesions show a thin fibrous cap (bracket) and a large necrotic core (arrow spans) black arrows indicating a necrotic core. Grade 3 lesions have very large necrotic cores. In this example extensive calcification is seen. Note point of fibrous cap rupture (arrow). Slides were subjected to hematoxylin and eosin staining, original magnification x 12.5.

c) Immunohistochemical detection of cathepsin B, cathepsin S and CD68. Cathepsin S, cathepsin B and CD68 were detected in slices of tissue imbedded in paraffin (n=21). Neighboring cross-sections of stable (Grade 1) and unstable (Grade 2 and 3) human carotid plaque were treated with indicated primary antibody followed secondary antibody. Expression of CD68 and cathepsin B were found to co-localize in stable (Grade 1) and unstable (Grade 2 and 3) regions of carotid plaque. While Cathepsin B was found overexpressed in all unstable plaques, cathepsin S expression, in contrast, was significantly elevated only in Grade 3 plaques.

Supplementary Figure III.

Supplementary Figure III. Probe labeled band identity
Lysates (150 μg) of human carotid plaque were labeled by 5μM GB123 probe for 30 min. then lysates were immunoprecipitated using anti-cathepsin B or anti-cathepsin S antibodies as indicated. Input and pellet samples were separated by SDS-PAGE and visualized by scanning of the gel with a Typhoon flatbed laser scanner.
Supplemental Figure IV. Cathepsin Protein levels in Plaques with Different Grades.
Western blot analysis of cathepsin B and cathepsin S extracted from same human carotid plaque samples as in (Figure 1a) Cathepsin S protein expression significantly increased in Grade 3 plaques and Cathepsin B is present in Grade 2 and 3 plaques but was lower in Grade 1 plaques.

Supplementary Figure V. Correlation of patient symptoms and plaque grade.
Plaques in each Grade were counted and determined 100%, the percent of Asymptomatic patients and Symptomatic Patients were calculated in each grade. An increase in the percent of symptomatic patients is detected as plaque progresses to Grade 3.
Supplementary Material

Supplementary Figure VI.

Supplementary Figure VI. Gene set enrichment analysis of genes involved in lysosomal activity. Expression profile of macrophages isolated from ruptured and stable human plaques (GSE41571) were analyzed by Gene Set Enrichment Analysis available from the BROAD Institute.
(A) Enrichment plot of genes involved in lysosomal activity are augmented in macrophages obtained from ruptured plaques (NES=2.918 and FDR < 0.001).
(B) Selected genes from KEGG lysosomal gene set are presented in heatmap.

Supplementary Figure VII.

Supplementary Figure VI: FACS dot blot analysis of macrophages markers in stable and unstable plaques. Lesion macrophages were isolated from OCT frozen stable and unstable plaques. Cells were co-stained with CD45/CD86 or CD45/MR, CD45 positive cells were gated and the percent of MR or CD86 positive cells were analyzed. a) A representative plaque forward scatter channel (FSC) area (A) vs. side scatter channel (SSC)-A.
b) To enrich analysis for macrophages, CD45+ cells were gated on, CD86 and MR expression was examined.
c) Dot blot analysis of cells treated with GB123, Cy5 signal and expression of CD45+MR+ (middle) or CD45+CD86+ cells (right) in human carotid plaques.
Supplemental Material

References

