Atherosclerosis and Lipoproteins

Destabilizing Role of Cathepsin S in Murine Atherosclerotic Plaques

Kenneth J. Rodgers, Deborah J. Watkins, Alastair L. Miller, Peter Y. Chan, Sharada Karanam, William H. Brissette, Clive J. Long, Christopher L. Jackson

Objective—Lysosomal proteinases have been implicated in a number of pathologies associated with extracellular matrix breakdown. Therefore, we investigated the possibility that the lysosomal proteinase cathepsin S may be involved in atherosclerotic plaque destabilization.

Methods and Results—Atherosclerotic plaques in the brachiocephalic arteries of fat-fed apolipoprotein E/cathepsin S double knockout mice had 73% fewer acute plaque ruptures (P=0.026) and were 46% smaller (P=0.025) than those in age-, strain-, and sex-matched apolipoprotein E single knockout controls. When the incidence of acute plaque rupture was normalized for plaque size, the reduction in the double knockouts was 72% (P=0.039). The number of buried fibrous layers, indicative of an unstable plaque phenotype, was reduced by 67% in the double knockouts (P=0.008). The cysteine proteinase inhibitor, egg white cystatin, was biotinylated and used as an active-site-directed probe for cathepsins. Biotinylated cystatin selectively detected cathepsin S in extracts of human carotid atherosclerotic plaque. Active cathepsin S was detectable in extracts of human atherosclerotic plaque but not in nondiseased carotid arteries. Active cathepsin S was especially prominent in macrophages in the shoulder regions of plaques, areas considered to be vulnerable to rupture. Cathepsin S protein colocalized with regions of elastin degradation in human coronary plaques.

Conclusion—These data provide direct evidence that an endogenous proteinase, cathepsin S, plays an important role in atherosclerotic plaque destabilization and rupture. (Arterioscler Thromb Vasc Biol. 2006;26:851-856.)

Key Words: atherosclerosis ■ plaque ■ pathology ■ cathepsin

The highly thrombogenic gruel in the core of an atherosclerotic plaque is luminally covered by a fibrous cap, weakening of which leads to plaque rupture and thrombus formation. Macrophages and T cells accumulate at sites of plaque disruption.1 Degradation of collagen by macrophage-derived matrix metalloproteinases has been reported,2 but less is known of other classes of proteinase, which may be released by activated macrophages.

The lysosomal cathepsins have been implicated in the development and progression of atherosclerosis. Increased levels of cathepsins F, K, and S are present in atherosclerotic lesions,3,4 whereas levels of the major extracellular inhibitor of cysteine proteinases, cystatin C, are decreased.5 In humans, an association has been shown between a genetically determined decrease in cystatin C levels and the severity of coronary artery disease.6

Cathepsin S/low-density lipoprotein (LDL) receptor double knockout mice have impaired atherogenesis when compared with LDL receptor single knockout controls.7 Grading of atherosclerosis in the aortic arch showed that there was a delay in lesion progression in the double knockouts. For example, 12 weeks of feeding atherogenic diet to the double knockouts resulted in an average lesion severity similar to that seen in single knockout controls after just 8 weeks of feeding, and 26 weeks of atherogenic diet feeding in the double knockouts produced lesions similar to those observed after 12 weeks in the single knockouts. This suggests that cathepsin S is involved in the rate of progression of lesions.

There is also evidence supporting a role for cathepsins in plaque destabilization. Human monocyte-derived macrophages secrete the proactive and active forms of cathepsins B, L, and S in vitro.8 Cathepsins S and K have similar elastolytic potency to neutrophil elastase at pH 7.5,9 and cathepsin K can cleave the triple helix of collagens I and III, so cathepsins can attack the structural proteins important for preservation of the integrity of the fibrous cap. Cathepsins have been detected extracellularly in plaques,10 further implicating them in fibrous cap degradation and plaque destabilization.

The development of an animal model of spontaneous plaque destabilization,11,12 and in particular the development...
of a model in which spontaneous plaque destabilization can be monitored in the short term,\textsuperscript{13} has made it possible to conduct intervention studies in which plaque stability is measured directly. In this model, plaques develop in the brachiocephalic artery of fat-fed apolipoprotein E (apoE) knockout mice. We tested the hypothesis that cathepsin S is involved in plaque destabilization by cross-breeding these mice with cathepsin S knockouts.\textsuperscript{14}

**Methods**

**Materials**
For materials, please see the online supplement, available at http://atvb.ahajournals.org.

**Animals**
Homozygous C57BL/6–129 apoE knockout mice and homozygous cathepsin S knockout mice on a pure DBA strain background\textsuperscript{12} were used. The initial pairings generated apoE\textsuperscript{−/−} cathepsin S\textsuperscript{−/−} double heterozygotes on a mixed C57BL/6–129–DBA strain background. After interbreeding of the double heterozygotes, male offspring were selected, and polymerase chain reaction analysis of tail-tip DNA was used to identify apoE\textsuperscript{−/−} cathepsin S\textsuperscript{−/−} double knockouts and their apoE\textsuperscript{−/−} cathepsin S\textsuperscript{+/−} single knockout controls, which were thus age and sex matched and on the C57BL/6–129–DBA mixed strain background. This approach resulted in random distribution of strain-related allelic variation between groups. The housing and care of the animals and all the procedures used in these studies were performed in accordance with the regulations of the University of Bristol and the United Kingdom home office.

At 6 to 8 weeks of age, male apoE\textsuperscript{−/−} cathepsin S\textsuperscript{−/−} double knockouts and apoE\textsuperscript{−/−} cathepsin S\textsuperscript{+/−} controls were fed high-fat rodent diet containing 21% pork lard and supplemented with 0.15% cholesterol (Special Diets Services) for 12 weeks. At termination, animals were exsanguinated and pressure perfusion fixed as described previously.\textsuperscript{12}

**Termination**
For termination, please see the online supplement.

**Mouse Plaques**

**Histology**
For histology, please see the online supplement.

**Morphological Analysis**
Sections stained for elastin were inspected for disruption of the cap of the plaque. Acute plaque disruption was accepted when a visible defect in the cap was accompanied by intrusion of erythrocytes into the region below it. In many animals, ≥1 smooth muscle cell– and elastin-rich layers, usually overlain with foam cells, were seen within the body of the plaque and were also counted.

**Morphometric Analysis**
For morphometric analysis, please see the online supplement.

**Lipid, Elastin, Collagen, and Macrophage Content**
For lipid, elastin, collagen, and macrophage content, please see the online supplement.

**Fibrous Cap Thickness**
For fibrous cap thickness, please see the online supplement.

**Human Plaques**
Human carotid endarterectomy specimens (n=20) were obtained within 1 hour of carotid endarterectomy surgery. Frozen sections of the specimens were examined qualitatively for the presence and extent of calcification, the size of the lipid core, and the thickness of the fibrous cap. Macroscopically normal (n=5) and diseased human coronary arteries (n=5) were obtained postmortem. The studies were approved by the institutional ethical review board, and subjects or their next-of-kin gave informed consent.

**Preparation of Biotinylated Cystatin**
For preparation of biotinylated cystatin (bCystatin), please see the online supplement. Egg white cystatin was biotinylated with N-biotinyl-L-aminocaproic acid N-hydroxysuccinimide ester. bCystatin was purified by column fractionation with monitoring of cathepsin S inhibitory activity using Z-Val-Val-Arg-7-amino-4-methylcoumarin (AMC) substrate.\textsuperscript{15}

**Immunohistochemistry**

**Mouse**
Some sections were developed by incubation with a goat polyclonal antibody to human cathepsin S or an isotype-matched nonimmune IgG control (1 μg/mL) overnight at 4°C. The secondary antibody was biotinylated rabbit anti-goat IgG (1:400; 30 minutes), developed using the Vectorstain ABC kit and DAB. The specificity of the anti–cathepsin S antibody was assessed by Western blotting using extracts of the J774 mouse macrophage cell line showing that only cathepsin S was recognized, and that there was no reaction with other proteins including cathepsins B, H, and L that are known to be expressed by J774 cells.\textsuperscript{16} For full details, please see the online supplement.

**Human**
For human immunohistochemistry, please see the online supplement. Cryosections of carotid endarterectomy plaque were incubated with bCystatin (60 μg/mL) for 60 minutes followed by a 30-minute incubation with Extravidin–horseradish peroxidase (HRP). Peroxidase activity was visualized using DAB. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide.

Sections developed with an antibody to human CD68 were blocked with goat serum and incubated with mouse anti-human CD68 (4 μg/mL) for 60 minutes. The secondary antibody was biotinylated goat anti-mouse IgG (1:1000), developed using Extravidin–HRP.

Cryosections of coronary plaque were blocked with rabbit serum in PBS containing pontamine sky blue 6BX (0.5%) for 30 minutes and incubated with goat anti-human cathepsin S (1 μg/mL). The secondary antibody was biotinylated rabbit anti-goat IgG (1:400), developed using Extravidin–fluorescein isothiocyanate. Sections were counterstained with propidium iodide.

**Statistical Analysis**
Values are expressed as mean±SEM. Data were analyzed using the programs InStat and Prism (GraphPad Software). A check was first made for similar variances and normality of distribution. If these were both passed, then an unpaired 2-sample 2-tailed Student t test was used. If the distributions were not normal, then a nonparametric Mann–Whitney test was used. If the normality test was passed but the variances differed significantly, then Welch corrected t test was used. Contingency data (presence or absence of acute plaque rupture) were analyzed by Fisher exact test. Discontinuous data (number of buried fibrous layers) were analyzed using the Mann–Whitney test. Significance was concluded when P<0.05.

**Results**

**Acute Plaque Rupture Is Inhibited in ApoE/Cathepsin S Double Knockout Mice**
The proportion of mice with acute plaque ruptures was decreased by 73% in apoE/cathepsin S double knockouts (P=0.026; Table 1).

**ApoE/Cathepsin S Double Knockout Mice Have a More Stable Plaque Phenotype**
The number of buried fibrous layers, a marker of an unstable plaque phenotype, was reduced by 67% from 1.33±0.29...
buried layers/mouse in apoE single knockout mice to 0.44±0.10 buried layers/mouse in double knockouts (P=0.008; Table 1).

The double knockouts had fibrous caps that were 70% thicker: 7.2±0.8 μm, compared with 4.2±0.7 μm in single knockout controls (P=0.020; Table 2). The analysis was complicated by the fact that the plaques in many of the double knockouts had not developed sufficiently to form a fibrous cap. All 9 of the apoE single knockout controls but only 12 of the 27 double knockouts had discernible fibrous caps (P=0.005), lending weight to the suggestion that cathepsin S fosters plaque development as well as destabilization.

Atherosclerotic Plaque Size Is Decreased in ApoE/Cathepsin S Double Knockout Mice

Plaque area in apoE single knockouts was 101.5±16.6 ×10^3 μm^2, but this was reduced by 46% to 55.1±9.9 ×10^3 μm^2 in double knockouts (P=0.025; Table 2). There were no significant differences in media or lumen area. Representative plaques are shown in Figure 1A and 1B. In apoE single knockout mice, cathepsin S immunopositivity was seen in the lipid-rich regions of plaques (Figure 1E).

The Increase in Plaque Stability in ApoE/Cathepsin S Double Knockout Mice Is Independent of the Reduction in Plaque Size

When the number of acute plaque ruptures was normalized by dividing by the plaque area, there was still a significant reduction, from 9.4±4.0 mm^2 in single knockouts to 2.7±1.4 mm^2 in double knockouts (−72%, P=0.039; Table 1). These data suggest that cathepsin S is a mediator of plaque destabilization.

Plaque Composition Is Altered in ApoE/Cathepsin S Double Knockout Mice

ApoE/cathepsin S double knockouts had 49% less plaque elastin than single knockout controls (controls 10.5±1.1%; double knockouts 5.3±1.3%; P=0.005). There were no significant differences in medial elastin, plaque collagen, plaque lipid, or macrophage content. There was no difference in the number of breaks in the medial elastic laminae (for full details, see Table II, available online at http://atvb.ahajournals.org).

bCystatin Selectively Detects Cathepsin S in Tissue Extracts

For full details, see the online supplement. On Western blots using bCystatin for detection, 1 major band was present in all of the plaque extracts (n=15, 6 of which are shown in Figure 2). No corresponding band was present in nondiseased carotids (n=5, 2 of which are shown in Figure 2). Using a polyclonal antibody, it was demonstrated that the major band corresponded to cathepsin S (Figure 2).

Cysteine Proteinase Activity Is Present in Human Atherosclerotic Plaques

Significant increases in bCystatin binding (P=0.002; Figure 3A) and the rate of hydrolysis of a cathepsin S substrate (P=0.002; Figure 3B) were found in extracts of carotid plaque when compared with extracts of control arterial tissue. Although there was appreciable variability in cysteine proteinase activity between specimens, this did not appear to be correlated with the stage of development of the plaque. All of the endarterectomy plaques examined were similar in terms of calcification, lipid content, and fibrous cap thickness. None showed characteristics associated with plaque instability or rupture.

Cysteine Proteinase Activity Localizes to Macrophages in Human Atherosclerotic Plaques

Cryosections of human atherosclerotic plaque tissue (n=20) were examined for enzymically active cysteine proteinase molecules using the bCystatin probe. Isolated groups of cells were found to be strongly positive, mainly in the shoulder regions of the plaque and at the edges of the lipid core (Figure 4A and 4B). When serial sections were examined by immunohistochemistry, these cells were all shown to be lipid-rich macrophages (Figure 4C and 4D). Incubation of tissue sections with cysteine proteinase inhibitors before incubation with bCystatin completely abolished the reactivity to bCystatin. Some extracellular cathepsin S immunopositivity was also seen; in coronary arteries, this appeared to be closely focused at areas of elastin fragmentation (Figure 4E). Adjacent regions of the same section with normal elastin were negative for cathepsin S (Figure 4F).

### TABLE 1. Plaque Stability in ApoE/Cathepsin S Double Knockout Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Acute Plaque Ruptures/Mouse</th>
<th>Buried Fibrous Caps/Mouse</th>
<th>Total Plaque Ruptures/Mouse</th>
<th>Acute Plaque Ruptures/mm^2 Plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE^-/- cathepsin S^-/- (n=9)</td>
<td>0.56±0.18</td>
<td>1.33±0.29</td>
<td>1.89±0.26</td>
<td>9.4±4.0</td>
</tr>
<tr>
<td>ApoE^-/- cathepsin S^-/- (n=27)</td>
<td>0.15±0.07*</td>
<td>0.44±0.10*</td>
<td>0.59±0.12*</td>
<td>2.7±1.4*</td>
</tr>
</tbody>
</table>

*P<0.05 vs apoE single knockout control.

### TABLE 2. Plaque Morphometric Data in ApoE/Cathepsin S Double Knockout Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Vessel Area (×10^3 μm^2)</th>
<th>Plaque Area (×10^3 μm^2)</th>
<th>Media Area (×10^3 μm^2)</th>
<th>Lumen Area (×10^3 μm^2)</th>
<th>Fibrous Cap Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE^-/- cathepsin S^-/- (n=9)</td>
<td>367.7±31.8</td>
<td>101.5±16.6</td>
<td>96.5±9.5</td>
<td>190.4±17.4</td>
<td>4.2±0.7</td>
</tr>
<tr>
<td>ApoE^-/- cathepsin S^-/- (n=27)</td>
<td>292.7±14.6*</td>
<td>55.1±9.9</td>
<td>77.4±5.5</td>
<td>164.2±7.6</td>
<td>7.2±0.8* (n=12)</td>
</tr>
</tbody>
</table>

*P<0.05 vs apoE single knockout control.
significantly smaller, they may simply have failed to develop reflection of impaired plaque progression? Because the direct that cathepsin S is involved in atherosclerotic plaque examined. The current study is therefore the first to show had smaller plaques, but plaque stability was not directly that cathepsin S null (\textit{H}11002). This was reduced by 73% in 0.56 acute plaque ruptures in the proximal part of the Control apoE single knockout mice experienced an average of 30% macrophage content in LDL receptor/cathepsin S double knockouts. The macrophage content was reduced from C, Ruptured plaque in the brachiocephalic artery of a male apoE single knockout mouse fed high-fat diet for 12 weeks. Stained with Miller elastin/van Gieson. Arrowhead indicates the site of rupture. D, Ruptured plaque in the brachiocephalic artery of a male apoE single knockout mouse fed high-fat diet for 12 weeks. Stained with Miller elastin/van Gieson. Arrowhead indicates the site of rupture. E, Immunostaining for cathepsin S in the brachiocephalic artery of a male apoE single knockout mouse fed high-fat diet for 12 weeks. Counterstained with methyl green, F, Cathepsin S immunostaining negative control with the primary anticathepsin S antibody replaced with an isotype-matched nonimmune IgG in the brachiocephalic artery of a male apoE single knockout mouse fed high-fat diet for 12 weeks. Counterstained with methyl green.

**Discussion**

Control apoE single knockout mice experienced an average of 0.56 acute plaque ruptures in the proximal part of the brachiocephalic artery (Table 1). This was reduced by 73% in animals that were additionally cathepsin S null (\textit{H}0.026), supporting the idea that this cysteine proteinase plays a role in atherosclerotic plaque destabilization. There was also a reduction in plaque size (\textit{H}46%; \textit{P}=0.025). In a previous study,7 cathepsin S/LDL receptor double knockout mice also had smaller plaques, but plaque stability was not directly examined. The current study is therefore the first to show directly that cathepsin S is involved in atherosclerotic plaque destabilization and rupture.

Is the reduction in the number of plaque ruptures just a reflection of impaired plaque progression? Because the plaques in the apoE/cathepsin S double knockouts were significantly smaller, they may simply have failed to develop to the stage at which instability could manifest itself. To address this, we normalized for plaque size by dividing the number of acute plaque ruptures by the plaque area. The normalized incidence of acute rupture was reduced by 71% in double knockouts (\textit{P}=0.039), showing that the reduction in plaque ruptures reflects a real change in plaque stability.

Plaques in the double knockout mice contained significantly less elastin, which is surprising given that cathepsin S is elastinolytic. However, it is consistent with a recent report that apoE knockout mice transplanted with cystatin C null bone marrow show an increase in plaque elastin.17 It is possible that cathepsin S is involved in the normal turnover of elastin during atherogenesis, and that intimal elastin deposition is compromised in its absence. In contrast, normal elastogenesis in the media was unaffected, and there was no change in the number of breaks in the medial elastic laminae. This is consistent with the lack of cathepsin S immunoreactivity in the media (Figure 1E).

In contrast to the report by Sukhova et al2 of a reduction in aortic arch plaque macrophage content in LDL receptor/cathepsin S double knockout mice, we saw no similar change in the brachiocephalic arteries of apoE/cathepsin S double knockouts. The macrophage content was reduced from \textit{\textless}30% to \textit{\textless}22% in the study of Sukhova et al2 but was only 19% in our single knockout control brachiocephalic arteries at a similar time point. At present, we are unable to explain the difference in macrophage accumulation between the 2 sites or why this should be cathepsin S sensitive in one but not the other.

The use of biotinylated inhibitors for the detection of active proteinases has so far been restricted to nitrocellulose membrane-bound proteinases after their separation by SDS/PAGE or isoelectric focusing.18,19 In the present study, methods have been developed for detecting active cysteine proteinases in cryosections of human tissue. This approach should be adaptable to proteinases of other classes provided that suitable inhibitors are available.

Because of similarities in their apparent molecular weights, it is difficult to separate cathepsins by 1D electrophoresis. Inactivation of other cathepsins by heating before electrophoresis allowed the selective detection of cathepsin S by bCystatin. If heat treatment was omitted, 3 bands were detected, the additional bands corresponding to cathepsins L and B (data not shown).
The lysosomal cysteine proteinases are optimally active at acidic pH. With the exception of cathepsin S, they are irreversibly inactivated after prolonged exposure to neutral pH or heating at neutral pH. The pH stability of cathepsin S has been used to increase the specificity of activity assays using peptide substrates. We took advantage of the pH stability of cathepsin S to allow its selective detection on affinity blots using bCystatin and developed a bCystatin binding assay to measure cathepsin S activity in tissue extracts. This correlated well with activity as assessed using a peptide substrate (see the online supplement). Increased cathepsin S activity was demonstrated in extracts of carotid plaques with both of these assays (Figure 3).

Because analysis of tissue extracts can only indicate gross changes in proteinase and proteinase inhibitor levels, we further used the active site-directed bCystatin probe to localize regions of active proteinase activity in tissue cryosections. Positive staining was located mainly between the fibrous cap and the lipid core and in the shoulder region of the plaque, areas vulnerable to rupture (Figure 4A and 4B). The positive cells were macrophage-derived foam cells (Figure 4D and 4E), supporting a link between the accumulation of modified lipids and proteins by macrophages and increased cathepsin activity. No positive staining with bCystatin was found in the sections from undiseased control carotid arteries. These data suggest that there is an increase in cysteine proteinase activity in human advanced carotid plaques, localized to specific regions rich in macrophage-derived foam cells. Cathepsin S protein also localized to these areas (Figure 1E). It has been reported previously that aortic sinus plaques in fat-fed apoE knockouts are primarily immunopositive for cathepsin S in the smooth muscle cell-rich fibrous cap and media. However, Sukhova et al found that in human plaques, cathepsin S protein was present in both foam cells (presumed to be macrophage derived) and smooth muscle cells, and that in more advanced plaques, cathepsin S immunopositivity in smooth muscle cells decreased. We reported previously that aortic sinus lesions in fat-fed apoE knockout mice do not rupture, so the lack of cathepsin S in plaque smooth muscle cells from our apoE knockout mice may reflect their more advanced phenotype.

In summary, both plaque size and the incidence of acute plaque rupture in the brachiocephalic arteries of fat-fed apoE/cathepsin S double knockout mice are reduced significantly. The reduction in the incidence of acute plaque rupture was independent of the effect on plaque size. This is the first direct demonstration that cathepsin S is involved in plaque rupture. A specific active site-directed probe was used to show that human carotid artery plaques contain enzymically active cysteine proteinases that are clustered in the shoulder regions and around the lipid core, localizing to lipid-rich macrophages. These data support the idea that cathepsin S
contributes to tissue destruction in the plaque and ultimately to atherosclerotic plaque rupture.

Acknowledgments

This work was supported by Pfizer (K.I.R.), Organon Laboratories Limited (D.J.W.), and the British Heart Foundation (A.L.M.). We gratefully acknowledge the assistance of the staff of the Vascular Studies Unit, Department of Surgery, Bristol Royal Infirmary, in obtaining carotid endarterectomy specimens.

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SUPPLEMENTARY MATERIAL

METHODS

Materials

Egg white cystatin, Extravidin™, avidin-alkaline phosphatase and avidin (monomeric)-agarose were purchased from Sigma-Aldrich (Poole, UK). D-biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester was from Boehringer Mannheim (Indianapolis, USA). Kaleidoscope Pre-stained Standards and an alkaline phosphatase, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) visualization kit were from Bio-Rad Laboratories (Hercules, USA). Sheep polyclonal antibodies to human cathepsin L and human cathepsin B were from Biogenes (Poole, UK). Z-Val-Val-Arg-AMC was purchased from Bachem (Witham, UK). Goat polyclonal antibody to cathepsin S was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Human recombinant cathepsin S was supplied by Pfizer Central Research (Sandwich, UK). Biotin-conjugated goat anti-rabbit immunoglobulin, streptABComplex/HRP and mouse anti-human CD-68 (Clone KP1) were from Dako (Ely, UK). Optimal cutting temperature (OCT) compound was purchased from BDH Laboratory Supplies (Poole, UK). The Vectastain ABC kit was from Vector Laboratories Ltd. (Peterborough, UK).
Termination

Animals were surgically anaesthetized by intraperitoneal injection of sodium pentobarbitone, before thorough terminal exsanguination by arterial perfusion via the abdominal aorta with phosphate-buffered saline (PBS) at a constant pressure of 100 mmHg, with outflow through the incised jugular veins. This was followed by constant pressure perfusion in situ with 10% formalin.

Histology

Brachiocephalic arteries were embedded in paraffin. Sectioning was started at the proximal end of the tissue, with a 3 µm section being taken for inspection as soon as the orifice of the brachiocephalic artery was reached. If that section did not show an acute plaque rupture, the sample was cut in for another 30 µm and then another 3 µm section taken for inspection. This process was repeated until either an acute plaque rupture was seen or the sample had been cut in to the 150 µm mark, whichever came first. Where an acute plaque rupture was seen all analyses were performed on serial sections at that location; if there was no acute plaque rupture, analyses were performed on serial sections previously taken at the 90 µm mark. The sites of analysis were recorded for each animal. For apoE single knockout controls, sections were taken at 73 ± 7 µm: for the apoE/cathepsin S double knockouts, at 89 ± 4 µm (no significant difference).

Morphometric analysis

One vessel cross-section was quantified per mouse. Analysis was performed using a computerised image analysis program (Image Pro Plus, Media Cybernetics, Carlsbad, USA). The lengths of the internal and external elasticae were recorded. These were used to derive the media area, by assuming them to be the circumferences of perfect circles. The plaque area
was measured directly, and was subtracted from the area enclosed by the internal elastic lamina to derive the true lumen area.

**Lipid content**

Plaque lipid content was determined by digitally processing colour images of delipidated paraffin sections into monochrome, causing tissue to be rendered as black and delipidated areas to appear as white. The total white area in the plaque was expressed as a percentage of the total plaque area to give the fractional lipid content.

**Elastin content**

Colour images of arterial sections stained with Miller’s elastin/van Gieson were analysed using Image Pro Plus. Pixels representing a medial elastic lamina were sampled, and then pixel threshholding was applied to measure the area occupied by elastin in both the media and the intima.

**Collagen content**

Colour images of arterial sections stained with picrosirius red were analysed using Image Pro Plus. Pixel threshholding was applied to measure the area occupied by collagen in the intima.

**Macrophage content**

Colour images of arterial sections immunostained with a purified rat monoclonal antibody against mouse macrophages (Mac2, 3.12 µg/mL; BD Biosciences, Oxford, UK), and developed with 3,3’-diaminobenzidine (DAB), were analysed using Image Pro Plus. Pixel threshholding was applied to measure the area occupied by macrophages in the intima.
**Fibrous cap thickness**

Fibrous cap thickness was determined at the thinnest part of the cap by computerised image analysis. The analysis was necessarily restricted to those plaques that had developed sufficiently to form caps.

**Human tissue extraction**

Atherosclerotic plaque tissue was obtained within one hour of carotid endarterectomy. The intimas were powdered under liquid nitrogen and extracted into 0.05 mol/L phosphate buffer, pH 6.0 containing 10 mmol/L sodium chloride, 0.1% Triton X-100 and the proteinase inhibitors pepstatin A (1 µmol/L), benzamidine (50 mmol/L) and ethylenediaminetetraacetic acid (EDTA; 2 mmol/L). Samples were centrifuged at 7,000 x g for 10 minutes and the protein content in the supernatant was measured. The pellet was washed with absolute ethanol, allowed to dry overnight at 37°C and the DNA content determined using the diaminobenzoic acid assay (1) with calf thymus DNA (1–50 µg) as standard.

**Electrophoresis**

Electrophoresis was carried out using 10% polyacrylamide gels with 2-(N-morpholino)ethane sulphonate/sodium dodecyl sulphate (MES/SDS) running buffer. Gels were either stained for 1 hour in 0.1% Coomassie R-250 in 40% methanol and 10% acetic acid and destained overnight in 10% ethanol/10% acetic acid, or proteins were electroblotted to nitrocellulose membranes using half-strength Towbin buffer (pH 8.3) (2). Samples were mixed with sample buffer giving a final concentration of 10% glycerol, 2.5% SDS, 0.001% bromophenol blue and 62.5 mmol/L Tris, pH 6.8. Samples were incubated for 10 minutes at either room temperature or 90°C.
Development of Western blots using polyclonal antibodies

Membranes were blocked overnight in 5% skim milk and then incubated overnight at 4°C with the antibody diluted in 3% skim milk. After washing, membranes were incubated with a 1:500 dilution of biotin-conjugated goat anti-rabbit IgG and then with avidin-alkaline phosphatase. Both were used at a 1:1000 dilution in 3% skim milk. Colour development was with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

Generation of the biotinylated cystatin active site probe

A 2-fold and a 20-fold molar excess of D-biotinyl-e-aminocaproic acid N-hydroxysuccinimide ester in dimethyl sulphoxide was mixed with a solution of cystatin (0.5 mg/mL) in phosphate-buffered saline (PBS) pH 7.5 at room temperature for 3 hours. Ammonium chloride (1 mol/L) was then added to the sample (10% v:v) and after 10 minutes the samples were dialyzed into PBS, pH 7.4.

The biotinylated cystatin (bCystatin) solution was applied to an avidin affinity column. Bound material was eluted with 2 mmol/L biotin in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS). Fractions were monitored for protein using the bicinchoninic acid procedure (3), and for inhibitory activity against cathepsin S. Cathepsin S inhibitory fractions were pooled and extensively dialyzed to remove free biotin. The avidin affinity-purified bCystatin was concentrated by diafiltration using a 10 kDa exclusion membrane and applied to a Superdex 75 column. The column was eluted and fractions were monitored for protein content and for inhibitory activity against cathepsin S.

The biotin content of the bCystatin samples and the number of free amino groups modified were determined using 2-(4'-hydroxyazobenzene) benzoic acid. Protein was
measured by the bicinchoninic acid procedure using bovine serum albumin (BSA) as standard.

Inhibitory activity against cathepsin S was determined using the substrate Z-Val-Val-Arg-AMC (4) and human recombinant cathepsin S. One unit of inhibitory activity was defined as the amount of inhibitor required to produce 50% inhibition of the activity of 1 µg of human recombinant cathepsin S.

**Development of blots using bCystatin**

Membranes were blocked overnight in 0.1% Tween in PBS (pH 6) and incubated for 1 hour with bCystatin (0.05 µg/mL) in PBS, 0.05% Tween 20 (pH 6). After washing, membranes were incubated with a 1:1000 dilution of avidin-alkaline phosphatase. Colour development was with NBT/BCIP.

**Biotinylated cystatin binding assay**

Tissue extracts were brought to pH 7.5 and incubated at 40°C for 60 minutes then allowed to bind to the wells of a 96 well microtitre plate overnight at 4°C. The wells were then blocked with 200 µL of 1% BSA in TBS for 1 hour and washed once with TBS containing 5 mmol/L dithiothreitol. Biotinylated cystatin in TBS was then added to the wells and allowed to bind for 2 hours at room temperature. The wells were washed three times with TBS and then 50 µL of Extravidin™-horseradish peroxidase (HRP) was added (1:1000 dilution) and left for 1 hour. The plate was washed again in TBS and then o-phenylenediamine was added. Colour was allowed to develop for 30 minutes and the absorbance at 450 nm was measured.
Determination of cathepsin S activity

Cathepsin S activity in tissue extracts was determined against the substrate Z-Val-Val-Arg-AMC (4). Assays were carried out in duplicate in 96 well microtitre plates in the presence of proteinase inhibitors at the following final concentrations: benzamidine (5 mmol/L), EDTA (5 mmol/L), phenylmethylsulphonyl fluoride (1 mmol/L), and pepstatin (1 µmol/L). Control wells contained E64 (10 µmol/L) and iodoacetic acid (100 µmol/L) in addition to the aforementioned inhibitors. Fluorescence was read with an excitation wavelength of 380 nm and an emission wavelength of 450 nm.
RESULTS

Characterisation of biotinylated cystatin

Biotinylated proteins were visualized using alkaline phosphatase/NBT/BCIP. In addition to cystatin (apparent molecular weight approximately 13 kDa), a number of other biotinylated proteins were also present (Figure I). After further purification by gel permeation chromatography a single biotinylated protein corresponding to bCystatin was present (Figure I).

In the bCystatin produced using a 1:20 molar ratio of cystatin to the biotin ester all 6 lysine residues of the cystatin were biotinylated, whereas at the 1:2 ratio an average of 2 lysine residues were biotinylated on each cystatin molecule (Table I). The higher level of biotin incorporation resulted in a reduction in the inhibitory activity of cystatin against cathepsins S (Table I) and L (results not shown), so the bCystatin synthesized using the 1:2 molar ratio was used in these studies.

bCystatin was used as a probe to detect active cysteine proteinases on Western blots of human atherosclerotic plaques, the sensitivity of the system being assessed against cathepsin B, L and S standards. As little as 5 ng of cathepsin S could be detected using the bCystatin probe (Figure II) but cathepsin B and L (up to 500 ng/lane) did not show any reactivity with this reagent on the affinity blots (data not shown). Using a modified protocol in which electrophoresis samples were left at room temperature for 10 minutes (rather than at 90°C for 10 minutes), cathepsin L could be detected at 500 ng per lane (Figure II) but cathepsin B could not be detected (not shown). This reflects the greater pH stability of cathepsin S compared to that of B or L, as well as the lower affinity of egg white cystatin for cathepsin B. Treatment of the membrane with E64, a synthetic cysteine proteinase inhibitor, prior to incubation with bCystatin blocked reactivity with cathepsin S (Figure II).
**Determination of cathepsin S activity**

A linear correlation was demonstrated between the proteolytic activity of cathepsin S and the binding of the biotinylated cystatin ($r^2=0.95$, p<0.001; Figure III).

**Plaque composition in apoE/cathepsin S double knockout mice**

ApoE/cathepsin S double knockouts had 49% less plaque elastin than single knockout controls (controls 10.5 ± 1.1%, double knockouts 5.3 ± 1.3%; p=0.005; Table II). There were no significant differences in medial elastin, plaque collagen, plaque lipid, or macrophage content. There was no difference in the number of breaks in the medial elastic laminae (Table II).

**Histochemistry: human**

Tissues were frozen under isopentane and embedded in optimum cutting temperature compound without fixation. Blocks of embedded tissue were stored at -70°C, and 5 µm cryosections were cut when required.

Cryosections of carotid plaque were blocked with 20% BSA and then incubated with bCystatin (60 µg/mL) for 60 minutes followed by a 30 minute incubation with Extravidin™-HRP. Peroxidase activity was visualized using diaminobenzidine as chromogen. Endogenous peroxidase activity in all sections was blocked with 3% hydrogen peroxide.

Sections developed with an antibody to human CD68 were blocked with goat serum and incubated with mouse anti-human CD68 (1:250) for 60 minutes. The secondary antibody was a biotinylated goat anti-mouse IgG (1:1000), developed using Extravidin™-HRP.

Cryosections of coronary plaque were blocked with rabbit serum in PBS containing pontamine sky blue 6BX (0.5%) for 30 min and incubated with goat anti-human cathepsin S
(1:100). The secondary antibody was a biotinylated rabbit anti-goat IgG (1:400), developed using Extravidin®-fluorescein isothiocyanate. Sections were counter-stained with propidium iodide. The specificity of this antibody was assessed by Western blotting, using extracts of the mouse J774 macrophage cell line and mouse liver (Figure IV).
References


## TABLE I

Characterisation of biotinylated cystatin

<table>
<thead>
<tr>
<th>Cystatin/Ester Ratio</th>
<th>Number of Lysine Residues Modified</th>
<th>Specific Activity (units/µg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>1:2</td>
<td>2.3</td>
<td>185</td>
</tr>
<tr>
<td>1:20</td>
<td>5.9</td>
<td>40</td>
</tr>
</tbody>
</table>

One unit is defined as the amount of inhibitor required to give 50% inhibition of 1 µg of human recombinant cathepsin S.
TABLE II

Vessel compositional data in apolipoprotein E/cathepsin S double knockout mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Plaque Lipid (%)</th>
<th>Plaque Macrophages (%)</th>
<th>Plaque Elastin (%)</th>
<th>Plaque Collagen (%)</th>
<th>Media Elastin (%)</th>
<th>Media Elastin Breaks (per mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt; Cathepsin S&lt;sup&gt;+/+&lt;/sup&gt; (n=9)</td>
<td>50.6 ± 2.3</td>
<td>18.5 ± 3.0</td>
<td>10.5 ± 1.1</td>
<td>45.1 ± 7.5</td>
<td>52.4 ± 9.3</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt; Cathepsin S&lt;sup&gt;-/-&lt;/sup&gt; (n=27)</td>
<td>54.1 ± 3.0</td>
<td>23.1 ± 3.7</td>
<td>5.3 ± 1.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25.5 ± 4.6</td>
<td>43.1 ± 3.5</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>*</sup>p<0.05 versus apolipoprotein E single knockout control.
FIGURE LEGENDS

Figure I: Western blot of biotinylated cystatin.
A molar ratio of 1:20 (cystatin:ester) was used to generate the samples run in lanes 1 and 5, and a ratio of 1:2 was used in samples run in lanes 2 and 4.

Lanes 1 and 2: Biotinylated cystatin samples following avidin affinity chromatography.
Lane 3: Standards with apparent molecular weights as indicated.
Lanes 4 and 5: Biotinylated cystatin samples after sequential avidin affinity chromatography and Superdex 75 FPLC.

Figure II: Affinity blot developed with biotinylated cystatin.

Lanes 1, 2 and 3: Cathepsin S (500 ng, 50 ng and 5 ng respectively).
Lane 4: Standards with apparent molecular weights as indicated.
Lane 5: Cathepsin S (500 ng); membrane pre-treated with E64 (10 µmol/L).

Lanes 6, 7 and 8: Cathepsin L (500 ng, 50 ng and 5 ng respectively).
Sample preparation was modified to avoid heating the samples to 90°C prior to electrophoresis.
Lane 9: Standards with apparent molecular weights as indicated.

Figure III: Comparison of inhibitor binding and peptidase activity in extracts of human artery.

Carotid plaques (n=6) and adjacent uninvolved carotid arteries (n=5) were extracted and assayed for both activity against the peptide substrate (Val-Leu-Leu-AMC) and also for binding of biotinylated cystatin. A strong correlation ($r^2=0.95$, $p<0.001$; Pearson’s test) was shown between the inhibitor binding and peptidase activity in the extracts. FU: fluorescence units.
Figure IV: Western blot of goat anti-human cathepsin S antibody.

Lane 1: Standards with apparent molecular weights as indicated.
Lane 2: Human recombinant cathepsin S.
Lane 3: Extract of the J774 mouse macrophage cell line (10 µL loaded).
Lane 4: Extract of the J774 mouse macrophage cell line (40 µL loaded).
FIGURE I

[Image of gel electrophoresis patterns with molecular weight markers labeled in kilodaltons (kDa): 75, 42.9, 31.8, 17.8, and 7.1.]
FIGURE IV

44.1KDa
32.7KDa
17.7KDa
7.1KDa

1 2 3 4