Cystatin C Deficiency Increases Elastic Lamina Degradation and Aortic Dilatation in Apolipoprotein E–Null Mice

Galina K. Sukhova, Bing Wang, Peter Libby, Jie-Hong Pan, Yaou Zhang, Anders Grubb, Kenneth Fang, Harold A. Chapman, Guo-Ping Shi

Abstract—The pathogenesis of atherosclerosis and abdominal aortic aneurysm involves substantial proteolysis of the arterial extracellular matrix. The lysosomal cysteine proteases can exert potent elastolytic and collagenolytic activity. Human atherosclerotic plaques have increased cysteine protease content and decreased levels of the endogenous inhibitor cystatin C, suggesting an imbalance that would favor matrix degradation in the arterial wall. This study tested directly the hypothesis that impaired expression of cystatin C alters arterial structure. Cystatin C–deficient mice (Cyst C−/−) were crossed with apolipoprotein E–deficient mice (ApoE−/−) to generate cystatin C and apolipoprotein E–double deficient mice (Cyst C−/−/ApoE−/−). After 12 weeks on an atherogenic diet, cystatin C deficiency yielded significantly increased tunica media elastic lamina fragmentation, decreased medial size, and increased smooth muscle cell and collagen content in aortic lesions of ApoE−/− mice. Cyst C−/−/ApoE−/− mice also showed dilated thoracic and abdominal aortae compared with control ApoE−/− mice, although atheroma lesion size, intimal macrophage accumulation, and lipid core size did not differ between these mice. These findings demonstrate directly the importance of cysteine protease/protease inhibitor balance in dysregulated arterial integrity and remodeling during experimental atherogenesis. (Circ Res. 2005;96:000-000.)

Key Words: cystatin C ■ apolipoprotein E ■ cysteine protease ■ atherosclerosis ■ smooth muscle cells

Atherosclerosis involves substantial remodeling of the arterial extracellular matrix.1 Considerable evidence links degradation of elastic laminae and of interstitial collagen with migration of leukocytes and smooth muscle cells (SMCs), neointima formation, atherosclerotic plaque rupture, and to arterial ectasia and aneurysm formation.

We previously proposed a role for cysteine proteases, in particular cathepsins S and K, in atherosclerosis. Human atheromata display substantially increased expression of cathepsins S and K compared with normal vessels, and cathepsin S–positive SMCs colocalize with sites of elastic lamina fragmentation in the tunica media.2 Human vascular SMCs and endothelial cells express cathepsin S–dependent elastolytic and collagenolytic activity in response to proinflammatory cytokines or growth factors in vitro.2,3 Our recent finding of attenuated atherosclerosis in cathepsin S–deficient mice provided direct evidence for cysteine protease involvement in atherogenesis.4 Mice deficient in cathepsin S had 50% less atherosclerosis than controls at both 8 and 12 weeks of atherogenic diet.

Importantly, we found a reciprocal relationship between the levels of cathepsins tested and their most abundant endogenous inhibitor cystatin C in diseased human arterial tissue. Both atherosclerotic and aneurysmal human lesions show reduction of cystatin C compared with normal arteries,5 indicating an imbalance in cysteine proteases and their inhibitor in human arterial disease.2 Eriksson et al6 recently demonstrated that a human cystatin C promoter polymorphism mutation, associated with reduced plasma cystatin C, correlated with a higher than average number of angiographically evident stenosis per coronary artery segment in survivors of myocardial infarction. Furthermore, Lindholt et al7 and our group5 independently showed a significant reduction of cystatin C level in the serum of patients with dilated abdominal aortas, highlighting the potential function of cystatin C not only in the local vascular microenvironment but systemically as well. Together, these findings suggest that cystatin C expression and its balance with its cognate proteases critically regulate arterial remodeling in atherosclerosis and aortic aneurysm progression. This study introduced inactivated cystatin C mutant alleles into apolipoprotein E–deficient (ApoE−/−) mice, which develop plaques that recapitulate many features of human atherogenesis,8 to test directly the importance of cysteine protease/cystatin C imbalance in atherogenesis and aortic ectasia.
Materials and Methods

Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup> Mice Generation and Animal Protocol

ApoE<sup>−/−</sup> (C57/B16) and Cyst C<sup>−/−</sup> mice (C57/B16/S129)<sup>9</sup> were crossbred to generate Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup> mice, which were interbred to generate ApoE<sup>−/−</sup>, Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup>, and Cyst C<sup>−/−</sup> littermates to avoid confounding caused by differences in genetic background. Six-week-old Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup>, ApoE<sup>−/−</sup>, and Cyst C<sup>−/−</sup> male mice consumed an atherogenic diet.<sup>10</sup> After 8 and 12 weeks of a “Western” type cholate-free diet,<sup>4</sup> mouse plasma was collected for plasma lipoprotein measurement and mouse aortae were harvested for lesion characterization, as described previously.<sup>4</sup>

Mouse Aorta Excision, Processing, and Evaluation

The aortic arch and abdominal aorta were prepared according to our standard protocol, as described previously.<sup>12–14</sup> Briefly, after opening the body, all mice were perfused at physiological pressure with normal saline via the left ventricle, and the adventitia was removed carefully before excising the heart-aorta complex (from the iliac bifurcation to the attached heart). The aortic arch was embedded in OCT for frozen section preparation.

Evaluation of atherosclerosis in mice used longitudinal sections of aortic arches as well as en face preparations of thoracic-abdominal aortae. Lesion size (intima and media), percentage of total area stained for SMCs, macrophages, interstitial collagen, and lipid of the aortic arch were analyzed as reported.<sup>4</sup> A segment of the lesser curvature of the aortic arch (3-mm) was defined by a perpendicular dropped from a fiducial point (the right side of the innominate artery ostium), as described previously.<sup>12</sup> To measure lesion sizes (intima and media), serial longitudinal sections of the aortic arch (30 × 6 μm/L) were analyzed microscopically and sections with maximal intimal thickness at the exact same location were used. Images captured by a digital imaging system were analyzed with Image-Pro Plus software (Media Cybernetics). The percentage of the total area with positive color was recorded for each section by two blinded observers. Data were presented as mean ± SD, and differences between groups were determined with a nonparametric Mann-Whitney test. Probability values < 0.05 were considered significant.

The entire thoracic-abdominal aorta, from aortic arch to bifurcation, was fixed in 4% paraformaldehyde, opened longitudinally, pinned out on a black wax surface, and stained with oil-red O.

Oil-Red O Staining for Lipids and Abdominal Aorta Expansion Measurement

To evaluate lipid accumulation in intimal lesions, frozen sections of aortic arches were incubated with 0.5% oil-red O solution. Slides were counterstained with Gill hematoxylin solution.

To measure thoracic-abdominal aorta lesion area and expansion, en face thoracic-abdominal aorta preparations were stained with oil-red O. The lesion area was determined as percent positive area after oil-red O staining. We captured images of all en face thoracic-abdominal aorta preparations were stained with Oil-Red O solution. The lesion area was determined as percent positive area after oil-red O staining.

Aortic SMC Isolation, Proliferation, Active Site-Labeling, and Activity Assay

Mouse SMCs were isolated from aortae.<sup>4</sup> SMC proliferation was determined using Celltiter 96 AQueous Solution according to the manufacturer’s instructions (Promega). A standard curve was created by assay of serial dilutions with known cell number.

Levels of active cysteine proteases in mouse aortic lesions and in isolated SMCs were examined with active site-labeling.<sup>15</sup> Briefly, 60 μg of protein extract from aortic tissue or 30 μg of protein from SMC lysates was incubated with [35S]-I-FPM for 1 hour and separated directly on 12% SDS-PAGE to visualize active cysteine protease signals.

To examine elastase activity in aortic tissues, protein extract (60 μg) was incubated with [3H]-elastin (200 μg) as described.<sup>15</sup> To determine elastase activity in live cells, equal numbers of SMCs (10<sup>5</sup>) from Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> mice were seeded into a 24-well plate. After growing to confluence, cells were incubated in serum-free medium for 24 hours, stimulated with interferon-γ, interleukin-1β, TNF-α, VEGF, or bFGF, all at 10 ng/mL, and incubated with [3H]-elastin (300 μg) for 48 hours. Soluble tritium released into the supernatant medium reflects elastolysis.<sup>2</sup> Data are expressed as μg of degraded elastin/10<sup>4</sup> cells per 24 hours.

To assess intracellular SMC elastase activity, SMCs were lysed by incubation in pH 5.5 lysis buffer.<sup>15</sup> An equal amount of protein (30 μg) was incubated with [3H]-elastin (300 μg) for 24 hours. Supernatants were collected and radioactivity determined.

SMC collagenase activity using fluorescein-labeled nonnatural collagen-type I (Calbiochem) was assayed by the same method as elastase activity. In a live cell collagenase assay, 0.3 mg of collagen was added to each well with culture medium (200 μL). In a cell lysis assay, collagen-type I (0.3 mg) was incubated with protein extract (30 μg) in pH 5.5 buffer. Digest collagen fragments were measured at 485 nm excitation and 530 nm emission.

Results

Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup> Mice Generation and Atherogenesis

This study used ApoE<sup>−/−</sup> and Cyst C<sup>−/−</sup> mice to generate Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup> breeding pairs for further interbreedings. The resulting Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup>, ApoE<sup>−/−</sup>, and Cyst C<sup>−/−</sup> littermates appeared normal in reproduction, body weight, and food/water consumption. To enhance atherogenesis, mice consumed the atherogenic diet for 8 or 12 weeks.<sup>16</sup> As in human atheroma, atherosclerotic lesions of ApoE<sup>−/−</sup> mice had reduced expression of cystatin C (not shown). As expected, total serum cholesterol in ApoE<sup>−/−</sup> mice that consumed the atherogenic diet exceeded that of those fed

Verhoeff-Van Gieson Elastin Staining and Picrosirius Red Collagen Staining

The Accustain Elastic Stain kit (Sigma) was used according to the manufacturer’s recommendations. Elastin preservation was graded as follows: grade 1, intact, well-organized elastic laminae; grade 2, elastic laminae with some interruptions and breaks; and grade 3, severe elastin fragmentation or loss.

Formalin-fixed frozen sections were incubated with Picrosirius red F3BA solution (Polysciences Inc) in saturated aqueous picric acid. Picrosirius red staining was analyzed by polarization microscopy.

Fibrous Cap Grading

We graded fibrous caps (FCs) according to their structure and presence/amount of SMCs and interstitial collagen. The grades were designated as follows: grade 0, no FC; grade 1, very thin and weak FC containing one layer of SMCs and almost no collagen; grade 2, FC consisting of 2 to 3 layers of SMCs, with some interstitial collagen; and grade 3, well-developed FC with multilayered SMCs and abundant fibrillar collagen.

Mouse Aortic SMC Isolation, Proliferation, Active Site-Labeling, and Activity Assay

Mouse SMCs were isolated from aortae.<sup>4</sup> SMC proliferation was determined using Celltiter 96 AQueous Solution according to the manufacturer’s instructions (Promega). A standard curve was created by assay of serial dilutions with known cell number.

Levels of active cysteine proteases in mouse aortic lesions and in isolated SMCs were examined with active site-labeling.<sup>15</sup> Briefly, 60 μg of protein extract from aortic tissue or 30 μg of protein from SMC lysates was incubated with [35S]-I-FPM for 1 hour and separated directly on 12% SDS-PAGE to visualize active cysteine protease signals.

To examine elastase activity in aortic tissues, protein extract (60 μg) was incubated with [3H]-elastin (200 μg) as described.<sup>15</sup> To determine elastase activity in live cells, equal numbers of SMCs (10<sup>5</sup>) from Cyst C<sup>−/−</sup>ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> mice were seeded into a 24-well plate. After growing to confluence, cells were incubated in serum-free medium for 24 hours, stimulated with interferon-γ, interleukin-1β, TNF-α, VEGF, or bFGF, all at 10 ng/mL, and incubated with [3H]-elastin (300 μg) for 48 hours. Soluble tritium released into the supernatant medium reflects elastolysis.<sup>2</sup> Data are expressed as μg of degraded elastin/10<sup>4</sup> cells per 24 hours.

To assess intracellular SMC elastase activity, SMCs were lysed by incubation in pH 5.5 lysis buffer.<sup>15</sup> An equal amount of protein (30 μg) was incubated with [3H]-elastin (300 μg) for 24 hours. Supernatants were collected and radioactivity determined.

SMC collagenase activity using fluorescein-labeled nonnatural collagen-type I (Calbiochem) was assayed by the same method as elastase activity. In a live cell collagenase assay, 0.3 mg of collagen was added to each well with culture medium (200 μL). In a cell lysis assay, collagen-type I (0.3 mg) was incubated with protein extract (30 μg) in pH 5.5 buffer. Digest collagen fragments were measured at 485 nm excitation and 530 nm emission.
normal chow (Table 1). Cyst C–/–ApoE–/– mice had modestly higher total cholesterol than did control mice when mice consumed the atherogenic diet for 8 or 12 weeks. Cystatin C–deficient mice had lower levels of high-density lipoprotein (HDL) at the 8-week time point (Table 1), whereas plasma triglyceride levels remained similar at both time points tested.

**Increased Elastic Lamina Degradation in Cyst C–/–ApoE–/– Mice**

We evaluated the degree of elastin degradation/fragmentation in longitudinal frozen sections from mouse aortae stained for elastica by Verhoeff-van Gieson (graded from 1 to 3, see Materials and Methods) (Figure 1A). Aortae from Cyst C–/–ApoE–/– mice showed significantly more elastic lamina fragmentation than those from ApoE–/– (2.7 ± 0.8 versus 1.5 ± 0.3; P < 0.001; Figure 1B) or Cyst C–/– mice (2.7 ± 0.8 versus 1.6 ± 0.2; P < 0.006).

Increased fragmentation of elastic laminae in cystatin C–deficient atherosclerotic lesions could reflect enhanced levels of cysteiny elastase activity. To test this hypothesis, we performed cysteine protease active site-labeling and elastase assay using protein extracts from freshly pulverized aortic tissue from Cyst C–/–ApoE–/– and ApoE–/– control mice. Tissue extract [125I]-JPM labeling allowed us to determine the amounts of active cysteine proteases. Cyst C–/–ApoE–/– aortic extracts showed much higher levels of cathepsins S and L, two potent mammalian elastases, as well as the long-chain active form of cathepsin B (∼31 kDa) (Figure 1C). Consistent with this observation, extracts of aortae from Cyst C–/–ApoE–/– mice had significantly higher ability to degrade 31 kDa) (Figure 1C). Consistent with this observation, extracts of aortae from Cyst C–/–ApoE–/– mice had significantly higher ability to degrade...
insoluble elastin than extracts from aortae of ApoE/H11002/H11002/H11002 mice wild type for Cyst C (Figure 1D).

Increased Intimal SMC and Collagen Content in Cyst C/H11546/H11546/H11546 ApoE/H11546/H11546/H11546 Mice
Fragmentation of elastic laminae in Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 mouse aortae might facilitate SMC migration from the tunica media and SMC accumulation in intimal lesions. To test this hypothesis, we stained sections of mouse aortae for α-actin (SMC) and Picrosirius red (collagen). Lesions from Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 mice had increased SMC content (% positive area) compared with ApoE/H11002/H11002/H11002 mice (9.4±3.6 versus 4.2±2.0; P<0.002; Figure 2). Increased collagen content (% positive area) in intimal lesions of Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 mice (14.4±4.9 versus 4.6±3.3; P<0.001; Figure 2) likely reflects accumulation of SMCs, the cell type responsible for the bulk of arterial extracellular matrix synthesis.

Increased SMC and collagen content in intimal lesions of Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 mice may promote fibrous cap formation. We examined fibrous caps in all mice and graded them according to their content of SMCs and collagen (see Materials and Methods). By these criteria, Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 mice had better developed fibrous caps than did ApoE/H11002/H11002/H11002 mice (Figure 2, bottom right panel; 1.7±0.9 versus 0.9±0.5; P<0.05).

We also evaluated macrophage, T cell, and lipid content as well as the percentage of intimal area occupied by the lipid core, using longitudinal sections of aortic arches. Of these variables, lipid content decreased in Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 intimal lesions compared with control (9.3±3% versus 15.2±6.6%; P=0.03), but the other features examined showed no significant change (macrophages, P<0.6; T cells, P<0.8; lipid cores, P<0.3).

Decreased Aortic Arch Tunica Media Thickness in Cyst C/H11546/H11546/H11546 ApoE/H11546/H11546/H11546 Mice
Intima and media thickness were measured on 3-mm segments of longitudinal sections of aortic arches stained for SMCs (α-actin) and macrophages (Mac-3).14 We detected no significant changes in aortic arch intimal size or lesion severity (Table 2). However, mice deficient in cystatin C had thinning of the tunica media in the aortic arch: 0.23±0.05 mm² in Cyst C/H11002/H11002/H11002 ApoE/H11002/H11002/H11002 mice versus 0.3±0.05 mm² in ApoE/H11002/H11002/H11002 mice (Table 2; P<0.005).
TABLE 2. Aortic Arch Lesion Characterization in Mice With 12 Weeks of Atherogenic Diet

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>n</th>
<th>Items</th>
<th>Measurement</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE−/−</td>
<td>11</td>
<td>Lesion grade*</td>
<td>3.8±1.2</td>
<td></td>
</tr>
<tr>
<td>Cyst C−/−ApoE−/−</td>
<td>10</td>
<td></td>
<td>4.0±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>11</td>
<td>Intimal size, mm²</td>
<td>0.3±0.2</td>
<td></td>
</tr>
<tr>
<td>Cyst C−/−ApoE−/−</td>
<td>10</td>
<td></td>
<td>0.3±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>11</td>
<td>Medial size, mm²</td>
<td>0.3±0.05</td>
<td></td>
</tr>
<tr>
<td>Cyst C−/−ApoE−/−</td>
<td>10</td>
<td></td>
<td>0.23±0.05</td>
<td>P&lt;0.005</td>
</tr>
</tbody>
</table>

*See Materials and Methods for scales used for grading.

Expanded Abdominal Aortae in Cyst C−/−ApoE−/− Mice

We found no significant difference in lipid deposition in abdominal aortae between Cyst C−/−ApoE−/− mice and their controls (% positive area, 9.2±4.8 versus 11±5.2; P=0.42), using an en face preparation of aorta explained previously.4,12,13 However, we detected significant expansion and elongation of the thoracic and abdominal aortae in Cyst C−/−ApoE−/− mice after 12 weeks of atherogenic diet. Both the aortic circumference at the level of the renal arteries (2.4±0.04 versus 2.1±0.05 mm; P<0.002) and the length of the aorta from the arch to the bifurcation (33±2.6 versus 30.5±1.7 mm; P<0.02) increased in ApoE−/− mice lacking cystatin C expression (Figure 3), but Cyst C−/−ApoE−/− mouse aortae (nonatherosclerotic) did not differ from ApoE−/− mice in either length (30.8±1.1 versus 30.5±1.7; P=0.55) or circumference (2.0±0.07 versus 2.1±0.05; P=0.67). The actual difference in aortic length and diameter between Cyst C−/−ApoE−/− and ApoE−/− or Cyst C−/−ApoE−/− mice may be underestimated because of postmortem aortic shrinkage, although all specimens were perfused at physiological pressure with saline for 3 to 5 minutes before excision, and were fixed immediately thereafter. Abdominal aortic expansion appeared quite consistent: 8 of 10 Cyst C−/−ApoE−/− mice had wider aortae (more than 2 mm), whereas only 1 of 11 ApoE−/− control mice reached this size. These observations may relate to the disrupted arterial elastin structure (Figure 1A and 1B), and the thinned tunica media detected in these mice (Table 2).

Regulation of Cysteine Protease Expression by Aortic SMCs

Both cathepsins S and K colocalized with SMCs at sites of internal elastic lamina fragmentation,2,4 regions of relatively deficient cystatin C expression.5 To test whether cystatin C regulates SMC cysteine protease activity, we performed [125I]-JPM active site-labeling using lysates of aortic SMCs isolated from Cyst C−/−ApoE−/− and control ApoE−/− mice. Quiescent SMCs showed no significant differences in expression of any known cysteine proteases, although cystatin C–deficient cells expressed more of a higher molecular weight cysteine protease (~45 kDa), possibly a cysteinyl cathepsin precursor (Figure 4A). However, Cyst C−/−ApoE−/− SMCs produced more active cathepsins B, L, and S than control cells after stimulation with proinflammatory cytokines or growth factors (Figure 4A). These findings suggest that cystatin C can influence cysteine protease expression and/or activity by vascular SMCs under inflammatory conditions.

SMC Elastolytic/Collagenolytic Activity and Proliferation

Cyst C deficiency also augmented the ability of aortic SMCs to degrade insoluble elastin in vitro. In fact, cultured Cyst C−/−ApoE−/− SMCs showed increased elastolytic and collagenolytic (against fluorescein-conjugated collagen-type I) activity. Cytokine or growth factor–treated or even unstimulated Cyst C−/−ApoE−/− SMCs exhibited enhanced levels of elastolytic/collagenolytic activity (Figure 4B and 4C). Although cell lysates from SMCs subjected to the same treatments also showed increased elastolytic/collagenolytic activity in Cyst C−/−ApoE−/− SMCs (not shown), the amount of extracellular matrix protein degraded was much lower, suggesting SMCs secrete the majority of the active elastolytic proteases.

At physiological concentrations, cystatin C inhibits T cell proliferation.12 Lack of cystatin C may thus alter vascular cell proliferation and further affect lesion cell content. Studies in mouse aortic SMCs tested this hypothesis. Cystatin C–deficient SMCs proliferated more rapidly than control cells (Figure 4D). If such increased SMC proliferation occurred in vivo it might contribute to the increased SMC and collagen content and fibrous cap development in Cyst C−/−ApoE−/− mouse atheroma (Figure 2).

Discussion

The ubiquitous protein cystatin C is the most efficient endogenous inhibitor known of cathepsins S, K, and L, potent mammalian elastases, and collagenases.18 We pre-

Figure 3. Aortic expansion in Cyst C−/−ApoE−/− mice. En face preparations of mouse aortae from aortic arch to bifurcation were stained with oil-red O. Aortic circumference (adjacent to the renal arteries) and length (from aortic arch to bifurcation) were both enlarged in Cyst C−/−ApoE−/− mice (n=10, open bars) compared with ApoE−/− mice (n=11, filled bars).
Previously detected significantly reduced expression of this protease inhibitor in human atherosclerotic and aneurysmal lesions compared with noninvolved normal arteries.\(^5\) Unlike the TIMPs, whose local levels change only modestly during atherogenesis,\(^{19–21}\) most SMC and macrophages in human atheromata lack cystatin C. However, both SMCs and macrophages in human atherosclerotic lesions express abundant cathepsins S and K,\(^2,5,5\) resulting in severe imbalance between cysteine proteases and their major inhibitor in these diseased arteries. As in the case of human atherosclerotic lesions, the atherosclerotic intima and the underlying tunica media in ApoE-deficient mice showed reduced cystatin C expression relative to normal aortae (not shown).

Although cystatin C–deficient mice show no overt abnormalities,\(^9\) mice lacking this protease inhibitor may have increased cysteine protease activity, especially animals with traumatic and/or other inflammatory conditions including atherogenesis. Some experiments in TIMP-deficient mice suggest such compensatory regulation. TIMP-3–deficient mice show increased MMP activity in the lung\(^{22}\) accompanied by spontaneous mouse airway enlargement.\(^23\) Deficiency of TIMP-1 led to significant loss of fibrillar collagens in mouse hearts, which amplified left ventricular remodeling during myocardial infarction\(^{24}\) and increased left ventricular end-diastolic volume and mass determined by echocardiography.\(^25\) These mice also had increased atheroma size, disrupted elastic laminae in the aortic tunica media, and developed more aortic medial ruptures on the ApoE\(^{-/-}\) background after consumption of an atherogenic diet.\(^{24,26}\) The aortae of these TIMP-1\(^{-/-}\)/ApoE\(^{-/-}\) animals showed particularly heightened MMP activity at sites of laminae elastin degradation.\(^27\)

As anticipated, cystatin C deficiency in ApoE\(^{-/-}\) mice yielded increased cysteiny1 elastase activity in aortae and disrupted arterial medial elastic laminae (Figure 1), similar to the sites of medial rupture in TIMP-1\(^{-/-}\)/ApoE\(^{-/-}\) mice,\(^{26}\) but MMP activities were not affected (data not shown). Higher levels of cysteine protease activity in Cyst C\(^{-/-}\)/ApoE\(^{-/-}\) mice could result from the absence of cystatin C as well as increased number of SMCs within the lesions, as these SMCs can express active elastolytic cysteine proteases (Figure 4A and 4B).\(^2\) This enhanced elastin fragmentation provides a potential mechanistic explanation for the increased SMC and collagen content of atherosclerotic lesions in these mice (Figure 2). Therefore, in normal arteries, abundant cystatin C, mostly produced by medial SMCs,\(^5\) participates in the homeostatic regulation of the integrity of the tunica media elastic laminae. The observed changes in aortic lesions of Cyst C\(^{-/-}\)/ApoE\(^{-/-}\) mice, ie, increased cysteine protease activity (Figure 1C), enhancement of elastin degradation (Figure 1B and 1D), SMC accumulation in the intima (Figure 2), and thinning of the arterial tunica media (Table 2), thus appear to result from absence of cystatin C, rather than altered MMP activities. Supporting this hypothesis, gelatin zymography of aortic extracts from Cyst C\(^{-/-}\)/ApoE\(^{-/-}\) mice showed no alteration of MMP activity relative to ApoE\(^{-/-}\) control lesions (data not shown). The present experiments provide direct support for this hypothesis. Cystatin C–deficient SMCs expressed higher cathepsin L and S activity after stimulation by proinflammatory cytokines or growth factors (Figure 4A) typically found in human atheroma.\(^{28}\) Consistent with human SMCs,\(^2\) these cells exhibited enhanced degradation of extracellular elastin (Figure 4B) and collagen type-I (Figure 4C), particularly after exposure to proinflammatory stimuli. Such increased cysteine protease activity and ECM degradation likely result from the lack of inhibitor.

Compared with cathepsin S–deficient mice,\(^4\) which showed reduced atherosclerotic lesion grades, decreased intimal lesion size, as well as lipid and macrophage content, we

---

**Figure 4.** Cystatin C deficiency influenced aortic SMC cysteine protease expression, activity, and proliferation. A, Cysteine protease active site-labeling. Increased expression of cathepsins L, B, and S was detected in stimulated SMC from Cyst C\(^{-/-}\)/ApoE\(^{-/-}\) mice compared with control cells. Asterisk indicates a higher molecular weight active cysteine protease that has not been characterized in this study. B and C, Elastase and collagenase assays. SMCs from Cyst C\(^{-/-}\)/ApoE\(^{-/-}\) mice showed greater activity in degrading both elastin (B) and collagen type-I (C). D, SMC proliferation assay. Two-fold serial dilutions starting from \(4 \times 10^3\) cells were incubated for 24 hours followed by proliferation assay. Cyst C\(^{-/-}\)/ApoE\(^{-/-}\) SMCs (open bars) proliferated more rapidly than ApoE\(^{-/-}\) cell (filled bars) in all dilutions. Data represent three independent assays.
detected no changes in these variables in cystatin C–deficient mice. Instead, these mice demonstrated reduced size of the tunica media (Table 2) and enlargement of the aorta (Figure 3), findings not observed in hypercholesterolemic cathepsin S–deficient mice.4 Therefore, absence of cystatin C may affect activity of several cysteine proteases (eg, cathepsins B and L) whose functions in atherogenesis remain undetermined.

It remains unexplained why lack of cystatin C did not affect the atherosclerotic lesion grades or intimal sizes (Table 2), but did alter serum lipid profiles (Table 1). HDL levels seem more sensitive to cystatin C deficiency in ApoE−/− mice on chow diet or at early (8 weeks) stage of plaque formation on atherogenic diet. However, total cholesterol and LDL levels are higher in mice that consumed atherogenic diet at both time points. Recent data demonstrated that cysteine proteases might participate in lipid metabolism.29,30 Incubation of mouse peritoneal macrophages with recombinant cathepsin F or S led to rapid loss of pre-β-HDL followed by cholesterol efflux reduction and lipid-free apoA-1 degradation in vitro.29 Further, cathepsins F, S, and K participate in LDL modification by degrading apoB-100, leading to enhanced LDL retention to human arterial proteoglycans.30 A similar function for cysteine proteases in metabolizing lipoproteins in the liver may exist, although no data are available to support this suggestion at present.

Lindholt et al and our laboratory have reported an inverse association of serum cystatin C levels with abdominal aortic aneurysm (AAA) progression,5,7 pointing to a role for cysteine proteases/cystatin C imbalance in aortic remodeling and expansion in humans. Systemic cystatin C deficiency in ApoE−/− mice recapitulated many features of human atherosclerotic aortic aneurysms, including severe medial elastic laminae degradation, tunica media thinning, and aortic ectasia,28 whereas cystatin C deficiency alone (Cyst C−/−/ApoE−/− mice) did not display these phenotypes. These results indicate a combined effect of altered proteolysis (Figures 1 and 4) and cholesterol homeostasis and inflammation8 in the pathogenesis of aortic aneurysms. Typical atherosclerotic aortic aneurysms occur regionally accompanied by signs of inflammation and enhanced protease expression. The more diffuse aortic ectasia and elongation reported here likely reflect systemic deficiency of cystatin C with enhanced proteolytic capacity against extracellular matrix proteins throughout the aorta, suggesting a role for cystatin C in aneurysmal dilatation. TIMP-1−/−/ApoE−/− mice showed similar findings.24,26 Deeficiency of TIMP-1 led to increased medial degradation, macrophage infiltration, and thoracic-abdominal aneurysm formation. Therefore, both MMPs and cysteine proteases contribute to degradation of the medial elastica and aneurysm formation (Figure 4).24,26

In conclusion, these data in genetically altered mice establish that cystatin C maintains elastic lamina integrity in the arterial tunica media. The multiplicity of proteases involved in aortic aneurysm formation and atherosclerosis suggested by the ensemble of these results require consideration when contemplating antiprotease treatments in the clinic.

Acknowledgments

This study is supported by NIH grants HL67249 (to G.K.S.), HL60942, HL67283 (to G.P.S.), HL67204 (to H.A.C.), HL56985, and HL05698508 (to P.L.) and from American Heart Association Grant-in-Aid 0355130Y (to G.P.S.). We thank Eugenia Shvartz for technical assistance and Karen Williams for editorial assistance.

References


Cystatin C Deficiency Increases Elastic Lamina Degradation and Aortic Dilatation in Apolipoprotein E-Null Mice
Galina K. Sukhova, Bing Wang, Peter Libby, Jie-Hong Pan, Yaou Zhang, Anders Grubb, Kenneth Fang, Harold A. Chapman and Guo-Ping Shi

Circ Res. published online January 13, 2005;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2005/01/13/01.RES.0000155964.34150.F7.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/