Lack of the Cysteine Protease Inhibitor Cystatin C Promotes Atherosclerosis in Apolipoprotein E–Deficient Mice

Eva Bengtsson, Fong To, Katarina Håkansson, Anders Grubb, Lena Bränén, Jan Nilsson, Stefan Jovinge

Objective—Degradation of extracellular matrix plays an important role in growth and destabilization of atherosclerotic plaques. Cystatin C, inhibitor of the collagen- and elastin-degrading cysteine proteases of the cathepsin family, is produced by virtually all cell types. It is present in the arterial wall but severely reduced in human atherosclerotic lesions.

Methods and Results—To determine the functional role of cystatin C in atherosclerosis, we crossed cystatin C–deficient (cysC−/−) mice with apolipoprotein E–deficient (apoE−/−) mice. After 25 weeks of atherogenic diet, mice lacking apoE and cystatin C (cysC−/− apoE−/−) had larger subvalvular plaques compared with cysC+/+ apoE−/− mice (766 000±20 000 μm² per section versus 662 000±19 000 μm² per section; P=0.001), suggesting an atheroprotective role of cystatin C. The plaques from cysC−/− apoE−/− mice were characterized by increased total macrophage content. To determine which cellular source is important for the antiatherosclerotic effect of cystatin C, we performed bone marrow transplantations. ApoE−/− mice were transplanted with either cysC−/− apoE−/− or cysC+/+ apoE−/− bone marrow. No significant differences in plaque area, macrophage, collagen, or lipid content of subvalvular lesions between the 2 groups were detected.

Conclusions—The result suggests that the protective role of cystatin C in atherosclerosis is dependent primarily on its expression in nonhematopoietic cell types. (Arterioscler Thromb Vasc Biol. 2005;25:2151-2156.)

Key Words: atherosclerosis ■ genetically altered mice ■ macrophages ■ cystatin C

Atherosclerosis is characterized by lipid accumulation in large and medium-sized arteries. Lipid accumulation and oxidation is followed by an inflammatory response and release of cytokines, which participate in the regulation of protease expression and activation. Proteases are involved in regulation of cell migration and plaque stability. Serine proteases and matrix metalloproteinases (MMPs) are the 2 most studied protease families. However, the cysteine proteases of the cathepsin family have been reported recently to play an important role in the atherosclerotic disease.

Cystatin C is a protein present in virtually all nucleated cells and is secreted to the extracellular space, working as an inhibitor of cysteine proteases of the cathepsin family. These enzymes are known to degrade elastin and collagen, the 2 major extracellular matrix constituents of the vascular wall. Cystatin C is present in substantial amounts in the normal vessel wall, but its concentration in human atherosclerotic lesions has been reported to be severely reduced. Cathepsins S and K are produced in human atheroma. In apolipoprotein E–deficient (apoE−/−) mice, mRNA levels of cystatin C do not differ between atherosclerotic lesions and nonaffected regions, whereas mRNA levels of several cathepsins are increased in lesions. Also, in the neointima formation of rabbits and rats, cathepsin expression was reported to be increased. Double knockout mice deficient in cathepsin S and low-density lipoprotein (LDL) receptor (LDLR) showed decreased atherosclerosis compared with control LDLR−/− mice.

To determine the role of cystatin C in atherosclerosis, we crossed cystatin C–deficient (cysC−/−) mice with apoE−/− mice. The result suggests that cystatin C has a protective role in atherogenesis because cystatin C deficiency results in larger lesion with increased macrophage content. To address the role of cystatin C in different cell types, we performed bone marrow transplantations.
continued for 25 weeks. Mice were euthanized by an intraperitoneal injection of an overdose of Hypnorm/Dormicum. Overnight, fasted mice were bled from the retro-orbital vein. Plasma cholesterol was analyzed with an enzymatic assay (Infinity cholesterol reagent kit; Sigma). All animal experiments were approved by the local animal care ethical committee.

Genotyping

Genotyping was performed with polymerase chain reaction (PCR) of DNA extracted from the tail. ApoE genotyping was performed using the primers Ex3ApoE (5′GAT GCC TAG CCG AGG GAG AGC CGG3′), In3ApoE (5′TGG TCC TCC CAT CCA GAA GCC3′), and NeoRev (5′TGC GCT GAC AGC CGG AAC AC3′), yielding a 330-bp band from apoE+/− mice and a 450-bp band from apoE−/− mice. Cystatin C genotyping was performed using the primers 5′AAA ACA AGG GCA TTC TCT ACA TAC3′, and 5′TTC TCT GTG CTT TAC GGT ATC G3′, followed by mouse adsorbed biotinylated rabbit anti-rat IgG antibodies against mouse cystatin C. Cystatin C was detected in plasma and in the aorta of cystC−/− apoE−/− mice. In cystC+/− apoE−/− mice, no cystatin C was present. Human cystatin C (hum cys C) was used as control.

Bone Marrow Transplantations

Ten-week-old female apoE−/− mice were used as recipient mice, subjected to 9.5-Gy potentially lethal total body irradiation and randomly assigned to receive bone marrow from 9- to 11-week-old cystC−/− apoE−/− or cystC+/− apoE−/− mice. Bone marrow was harvested by flushing the femurs and tibias of the donor mice, red blood cells were lysed in 0.2% NaCl at room temperature for 5 minutes, and bone marrow cells were washed and resuspended in PBS. Recipient mice were injected with 2×106 bone marrow cells in 0.2 mL through the tail vein. One day before and 7 days after the bone marrow transplantation, recipient mice were given 2 mg/mL neomycin sulfate (Sigma) in drinking water. Confirmation of transplantation was performed with PCR analysis of blood cells lysed in 10 mmol/L Tris-HCl, pH 8.0, 1% Tween-20, and digested with 400 μg/mL protease K at 60°C for 2 hours. Primers specific for the male Y chromosome of the donor mice were used.13 Two weeks after transplantation, mice were given a high-fat diet for 24 weeks.

Histological Analysis

Microscopic dissection of the heart and aorta was performed. The heart and proximal aorta were fixed in Histochoice, embedded in Tissue-Tek (Sakura), and collected in series consisting of 4 glasses with 5 10-μm cryosections on each glass. Seres from the aortic valves were stained with Oil Red O technique for lipid content and with Masson’s technique (Fuchsine-Ponceau from Chroma-Biosciences). Human cystatin C (Calbiochem) was used as control.

Plasma Cholesterol Levels

Cholesterol levels in plasma of the mice were assayed by colorimetric assay. Samples from cystC−/− apoE−/− mice contained higher levels of cholesterol than samples from cystatin C–expressing mice (908±68 mg/dL and 605±57 mg/dL, respectively; P=0.002).

Effect of Cystatin C Deficiency on Atherosclerotic Lesion Formation

Cross-sections from lesions in the aortic valve region were stained with hematoxylin and the lesion area quantified. Plaques from cystC−/− mice were significantly larger than plaques from mice expressing cystatin C (766 000±20 000 μm² per section and 662 000±19 000 μm² per section, respectively; P<0.001; Figure 2A). Plaques detected by Oil Red O staining of en face preparations of the descending aorta tended to be increased in cystC−/− mice (Figure 2B) even if it did not reach the level of predefined statistical significance.

To determine whether the increase in lesion areas in cystC−/− mice was a secondary effect attributable to the higher cholesterol levels, we performed correlation analysis. There
was no significant correlation between plasma cholesterol levels and plaque area in either of the groups (cysC<sup>−/−</sup>-apoE<sup>−/−</sup> vs. apoE<sup>−/−</sup>, r = −0.025, P = 0.93; cysC<sup>−/−</sup>-apoE<sup>−/−</sup> vs. apoE<sup>−/−</sup>, r = −0.24, P = 0.37). Moreover, a significant correlation between the plaque areas and the genotype of the mice was found even after controlling for plasma cholesterol levels.

### Effect of Cystatin C Deficiency on Inflammatory Cells, Collagen Content, and Aortic Expansion

To evaluate whether lack of cystatin C affects the inflammatory process of atherosclerosis, lesions in the aortic valves were stained for monocyte/macrophage content. The relative proportion of macrophages was not significantly different in cysC<sup>−/−</sup>-apoE<sup>−/−</sup> mice and cysC<sup>+/+</sup>-apoE<sup>−/−</sup> mice (Figure 3A). However, the total amount of macrophages was increased in cysC<sup>−/−</sup>-apoE<sup>−/−</sup> mice compared with cysC<sup>+/+</sup>-apoE<sup>−/−</sup> mice, (183 000 ± 22 000 μm<sup>2</sup> per section and 126 000 ± 10 000 μm<sup>2</sup> per section, respectively; P = 0.027). Subvalvular lesions were stained with Masson’s technique to determine whether lack of cystatin C and the resulting potential increase in cathepsin activity would affect the collagen content of the plaques. No significant difference was detected in collagen content in cysC<sup>−/−</sup>- mice compared with mice expressing the protein (Figure 3B).

Because cystatin C is an inhibitor of elastin-degrading cathepsins, a potential increased elastase activity in the cysC<sup>−/−</sup>- mice could be expected. A recent publication reported increased aortic expansion in apoE<sup>−/−</sup> mice lacking cystatin C expression after 12 weeks of Western diet, which may be attributable to a disrupted arterial elastin structure. Thus, we measured the aortic circumferences at 5-mm intervals of the aorta, starting at the aortic arch and ending at the bifurcation, but could not detect any significant difference between cysC<sup>−/−</sup>- apoE<sup>−/−</sup> and cysC<sup>+/+</sup>- apoE<sup>−/−</sup> mice (Figure 4). Moreover, the length of the aorta did not differ between cysC<sup>−/−</sup>- and cysC<sup>+/+</sup>- mice (27.1 ± 1.1 and 29.0 ± 0.3 mm, respectively; P = 0.15).

### Effect of Transplantations of CysC<sup>−/−</sup>- Hematopoietic Cells

To determine whether the increased atherosclerotic manifestations of cysC<sup>−/−</sup>- mice were attributable to lack of cystatin C in hematopoietic cells, such as macrophages, lymphocytes, and platelets, we performed bone marrow transplantations. Ten-week-old female apoE<sup>−/−</sup> mice were injected with either cysC<sup>−/−</sup>- apoE<sup>−/−</sup> or cysC<sup>+/+</sup>- apoE<sup>−/−</sup> bone marrow from male donor mice of similar age. The mice were given high-fat diet for the same time period as the double knockout mice. To establish engraftment of donor cells, blood cells of recipient mice were analyzed for presence of the Y chromosome (Figure 5). In addition, a PCR-confirming presence of the
cysC−/− or cysC+/+ gene in blood cells of the mice was performed (data not shown). The bone marrow–transplanted mice showed no differences in weight or plasma cholesterol levels (301±34 and 377±50 mg/dL, transplanted with cysC−/− or cysC+/+ bone marrow cells, respectively; P=0.22).

The extent of atherosclerosis was analyzed by measuring cross-sections of subvalvular plaque area as well as lipid accumulation of the thoracic part of the aorta in en face preparations. However, no significant differences were found in apoE−/− mice, which received cysC−/− apoE−/− bone marrow cells compared with mice injected with cysC+/+ apoE−/− cells (Table). In addition, lipid content in subvalvular lesions, determined by Oil Red O staining, was similar in the 2 groups (Table).

The ability of inflammatory cells to degrade the basement membrane and extracellular matrix components present in the intima is likely to be affected by protease activity. Correspondingly, an increased amount of macrophages was seen in apoE−/− mice lacking cystatin C. Thus, we looked for differences in macrophage content in lesions of bone marrow–transplanted mice as a measurement of monocyte/macrophage infiltration. However, no such difference was found (Table). As a measurement of macrophage protease activity on connective tissue composition, Masson’s staining of the lesions was quantified. The collagen content of lesions did not differ between the 2 groups (Table).

**Discussion**

Protease inhibitors are generally regarded as atheroprotective because proteases participate in matrix degradation, a process regarded mostly as atherogenic. Matrix degradation facilitates macrophage infiltration. Moreover, degradation of the fibrous cap may result in plaque rupture. On the other hand, smooth muscle cell (SMC) migration from the media to the intima also requires degradation of matrix proteins of surrounding basement membranes as well as the internal elastic lamina. This process is believed to stabilize the plaque, although plaque area increases. In addition, degradation of matrix components binding LDL may influence the disease progression. The complex role of proteases is exemplified by studies showing that MMP-3 deficiency in apoE−/− mice resulted in increased lesion area, whereas lack of MMP-9 reduced plaque area, and lack of MMP-12 did not affect lesion growth.18,19

The question of whether the protease inhibitor cystatin C is an atherogenic or protective protein is difficult to answer without performing in vivo studies. However, lack of the protease cathepsin S, which is inhibited by cystatin C, in LDLR−/− mice decreases atherosclerosis,11 thus pointing for a role of cystatin C as an atheroprotective protein. To directly address the role of cystatin C in cardiovascular disease, we generated atherosclerotic cysC−/− apoE−/− mice and compared them with cysC+/− apoE−/− mice. In apoE−/− mice, cystatin C deficiency results in an increased subvalvular plaque area as well as a tendency toward increased plaques in the aorta, demonstrating an atheroprotective role of cystatin C. The increase in lesion area in the subvalvular area was small (15%) but highly significant (P=0.001; Figure 2A). The increase in aortic atherosclerosis was greater (2-fold) but did not reach significance. This disparity could be because aortic valves are one of the earliest sites in the aortic tree where lesions will appear, whereas lesions in the descending aorta form at a later time point.

The reason for the increased plasma cholesterol of cysC−/− apoE−/− mice compared with cysC+/− apoE−/− mice remains

### Histological Analysis of Bone Marrow–Transplanted Mice

<table>
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<tr>
<th>Analysis</th>
<th>Male Bone Marrow Donor</th>
<th>Female Bone Marrow Donor</th>
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</thead>
<tbody>
<tr>
<td><strong>ApoE−/− CysC−/− Mice</strong></td>
<td><strong>ApoE−/− CysC+/+ Mice</strong></td>
<td><strong>ApoE−/− CysC−/− Mice</strong></td>
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<td><strong>n=11</strong></td>
<td><strong>n=13</strong></td>
<td><strong>n=13</strong></td>
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<td><strong>P</strong></td>
<td><strong>P</strong></td>
<td><strong>P</strong></td>
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<td>Cross-sectional lesion area, µm²/section</td>
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<td>729 000±52 000</td>
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<tr>
<td>Lipid en face prep, % of vessel area</td>
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<td>3.76±0.61</td>
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<td>Lipid, % of plaque area</td>
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<td>Macrophage, % of plaque area</td>
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<tr>
<td>Collagen, % of plaque area</td>
<td>17.2±1.7</td>
<td>19.4±2.3</td>
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Female apoE−/− mice were transplanted with bone marrow from male cysC−/− apoE−/− or cysC+/+ apoE−/− mice. Cross-sections of subvalvular lesions were stained and quantified for lipid, macrophage, and collagen content as well as lesion area. En face preparations of the aorta were stained and quantified for lipid content.
to be identified. No studies have reported that cystatin C levels in plasma are correlated to cholesterol levels. Several cathepsins (S, K, and F) have been shown to degrade apoA-I present on high-density lipoprotein particles and have an effect on its cholesterol acceptor function and its ability to induce cholesterol efflux in macrophages.

Subvalvular lesions showed an absolute increase in macrophage content in mice lacking cystatin C, suggesting a role for cystatin C in infiltration of inflammatory cells or protease activity of macrophages. Thus, we performed bone marrow transplantations to determine whether the atheroprotective effect of cystatin C was attributable to lack of cystatin C in hematopoietic cells. The bone marrow–transplanted mice were given a high-fat diet for 24 weeks to enable a comparison with the double knockout mice. The bone marrow–transplanted animals had lower cholesterol levels compared with nontransplanted animals. We previously noted the same effect in a previous study, and van Eck et al reported lower triglyceride levels up to 10 weeks after bone marrow transplantation.

Cystatin C deficiency in bone marrow–derived cells had no effect on plaque area, lipid accumulation, or macrophage or collagen content in the lesions (Table). This observation suggests that nonhematopoietic cells in the artery wall, such as SMCs or endothelial cells, produce the cystatin C that acts to suppress plaque development. However, it cannot be excluded that the lack of effect could be the result of a downregulation of cystatin C in activated macrophages, which could minimize the effect of cystatin C deficiency in macrophages.

The increased presence of macrophages in the cysC−/− apoE−/− mice may be explained by the absence of cystatin C in endothelial cells. A recent publication showed that human endothelial cells express cystatin C. However, whether lack of cystatin C in these cells results in an elevated expression of adhesion molecules attracting inflammatory cells remains to be shown.

During preparation of this manuscript, a publication presented cysC−/− apoE−/− mice. These male mice were analyzed after 8 and 12 weeks of high-fat diet. The cysC−/− mice exhibited increased elastic lamina degradation and aortic dilatation compared with cysC+/+ apoE−/− mice, whereas lipid cores or lesion size did not differ. Moreover, increased SMCs and collagen content of the lesions were described. The observed differences from the study presented here may be explained by the shorter time period of high-fat diet, which was less than half of our study. Differences in plaque size may increase with time and may therefore only be seen at later stages of the disease. Also, the sex of the mice will affect the progression of the disease, as reported by Caligiuri et al. Our study was performed on female mice, which develop lesions at an earlier age than male mice, whereas Sukhova’s newly published study assessed lesion formation on male mice. In addition, the location of the lesion is important (eg, Schiller et al reported contrasting results in bone marrow–transplanted LDLR−/− mice on a high-fat diet). In their study, aortic lesion areas were larger in bone marrow–transplanted mice compared with nontransplanted mice when assessing thoracic aortas, whereas the opposite result was seen when lesions in the aortic valves were analyzed.

In our study, we compared lesions in the aortic valves, one of the earliest sites of the arterial tree with atherosclerotic lesions, whereas Sukhova et al assessed lesions in the aortic arch.

Altogether, the differences in our study and Sukhova’s regarding lesion size and composition are most probably attributable to differences in the length of high-fat diet of the mice, sex of the mice, and anatomic site of analyzed lesions. The fact that the mice in our study were given a high-fat diet for 25 weeks compared with 12 weeks in Sukhova’s study, were female mice instead of male mice, and lesions in the aortic valves were analyzed instead of lesions in the aortic arch, indicates that the lesions in our study will be at a more advanced stage of atherogenesis than the lesions analyzed in Sukhova’s study.

In summary, we conclude that cystatin C has an antiatherosclerotic function in apoE−/− mice, and that absence of cystatin C results in larger lesions at later stages of atherosclerosis. This effect is not dependent on cystatin C in hematopoietic cell types because transplantations with cysC−/− bone marrow cells had no effect on lesion area or plaque composition. Instead, the protective effect of cystatin C most likely has its origin in other cell types present in aortic lesions, such as SMCs and endothelial cells, both known to produce cystatin C.

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