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Cathepsin L Deficiency Reduces Diet-Induced Atherosclerosis in Low-Density Lipoprotein Receptor–Knockout Mice

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Background—Remodeling of the arterial extracellular matrix participates importantly in atherogenesis and plaque complication. Increased expression of the elastinolytic and collagenolytic enzyme cathepsin L (Cat L) in human atherosclerotic lesions suggests its participation in these processes, a hypothesis tested here in mice.

Methods and Results—We generated Cat L and low-density lipoprotein receptor (LDLr) double-deficient (LDLr−/−Cat L−/−) mice by crossbreeding Cat L–null (Cat L−/−) and LDLr-deficient (LDLr−/−) mice. After 12 and 26 weeks of a Western diet, LDLr−/−Cat L−/− mice had significantly smaller atherosclerotic lesions and lipid cores compared with littermate control LDLr−/−Cat L+/− and LDLr−/−Cat L+/+ mice. In addition, lesions from the compound mutant mice showed significantly reduced levels of collagen, medial elastin degradation, CD4+ T cells, macrophages, and smooth muscle cells. Mechanistic studies showed that Cat L contributes to the degradation of extracellular matrix elastin and collagen by aortic smooth muscle cells. Smooth muscle cells from LDLr−/−Cat L−/− mice or those treated with a Cat L–selective inhibitor demonstrated significantly less degradation of elastin and collagen and delayed transmigration through elastin in vitro. Cat L deficiency also significantly impaired monocyte and T-lymphocyte transmigration through a collagen matrix in vitro, suggesting that blood-borne leukocyte penetration through the arterial basement membrane requires Cat L. Cysteine protease active site labeling demonstrated that Cat L deficiency did not affect the activity of other atherosclerosis-associated cathepsins in aortic smooth muscle cells and macrophages.

Conclusions—Cat L directly participates in atherosclerosis by degrading elastin and collagen and regulates blood-borne leukocyte transmigration and lesion progression. (Circulation. 2007;115:2065-2075.)

Key Words: atherosclerosis ■ cathepsin L ■ collagen ■ elastin ■ metalloproteinases ■ receptors, LDL ■ remodeling

Atherosclerosis involves substantial remodeling of the vascular adventitia, mediated by matrix metalloproteinases (MMPs), serine proteases, and the lysosomal cysteine proteases cathepsins (Cats).1–5 Because earlier studies demonstrated that Cat S and K, enzymes that possess strong elastinolytic and collagenolytic activities, are abundant in human atherosclerotic lesions, we hypothesized that these proteases participate importantly in atherogenesis.4 Indeed, recent studies comparing low-density lipoprotein receptor (LDLr)–deficient (LDLr−/−) and apolipoprotein E–null (Apoe−/−) mice showed that Cat S deficiency reduced atherosclerosis in LDLr−/− mice by 30% and 50% after 26 and 12 weeks of a Western diet, respectively.5 Cat K–deficient Apoe−/− mice showed similar reductions, although different mechanisms may pertain.6 Although Cat S contributes to atherogenesis by degrading medial elastica laminae and promoting leukocyte transmigration through the subendothelial basement membrane, Cat K appears to act mainly on macrophage foam cell formation.

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We recently demonstrated increased expression of Cat L, another member of the cysteine protease family potentially important for atherosclerosis because of its elastinolytic and collagenolytic activities,7 in human atherosclerotic lesions.8 All major cell types in human atheroma, including smooth muscle cells (SMCs), endothelial cells (ECs), and macrophages, express Cat L protein and activity. More important, patients with coronary artery stenosis had >2-fold-higher serum Cat L levels than patients lacking stenotic lesions. In addition, serum levels of Cat L correlated significantly with the percentage of coronary artery stenosis in a cohort of 319

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patients, suggesting involvement of Cat L in human atherogenesis. The present study tested the hypothesis that Cat L contributes importantly to aspects of atherogenesis in genetically altered mice.

Methods

**LDLR⁻/⁻ Cat L⁻/⁻ Mice Generation and Atherosclerotic Lesion Characterization**

LDLR⁻/⁻ mice (C57/BL6, The Jackson Laboratory, Bar Harbor, Maine) and Cat L⁻/⁻ mice (C57/BL6/129) were crossedbreed to generate LDLR⁻/⁻ Cat L⁻/⁻ breeding pairs. The resulting male LDLR⁻/⁻ Cat L⁻/⁻, LDLR⁻/⁻ Cat L⁺/⁺, and LDLR⁺/⁺ Cat L⁻/⁻ mice consumed a Western diet (Research Diet Inc, New Brunswick, NJ) for 12 or 26 weeks to produce atherosclerosis, followed by lesion characterization as described previously. We analyzed mouse atherosclerotic lesions on a 3-mm segment of the lesser curvature of the longitudinal sections from aortic arch (defined by a perpendicular line dropped from the right side of the innominate artery) using previously published approaches. Frozen sections (6 µm) of mouse aortic arches were prepared. Although a total of ~30 sections were made from each arch, those containing all 3 branches and maximal lumen diameter are limited. For lesion characterization, we selected 10 to 15 serial sections (6 µm) from the designed area in the aortic arch. One section per mouse was used for each of the immunological and histological stainings listed below: macrophages (mac-3; Pharmingen, San Diego, Calif; 1:1000), T cells (CD4; Pharmingen; 1:100), SMCs (α-actin; Santa Cruz Biotechnology Inc, Santa Cruz, Calif; 1:75), cell apoptosis (TUNEL assay; Roche, Basel, Switzerland), cell proliferation (Ki67; Sigma Chemical Co, St Louis, Mo; 1:100), lipid (0.5% Oil-Red O; Sigma), collagen (0.1% Sirius Red F3BA; Polysciences Inc, Warrington, Pa), and elastin (Verhoeff-van Gieson; Accustain Elastin Stain Kit; Sigma). Lesion cell contents were determined either by counting the cells (T cells) in the intimal lesions or by measuring the percentage of positive area (SMCs) or the absolute positive area (macrophages) in the intimal area. Intimal and medial sizes and lipid core areas were determined as described previously. Medial elastica degradation also was graded as previously described.11 Lesion sizes were graded as described12 using the following grading key: grade 0, no lesion; grade 1, few macrophages underlying endothelium; grade 2, fatty streak–like lesion; and grades 3 through 5, advanced lesions containing lipid cores and high levels of macrophages. To measure serum lipid profiles, developed lesions and higher grades to advanced lesions containing lipid containing 10% fetal bovine serum. After removal of EC culture media, CD4⁺ T cells were separated from positive areas by depleting antigen-presenting cells and CD8⁺ T cells with I-A⁻ and CD8 antibodies (Pharmingen), respectively, followed by complement lysis.14

A 96-well chemotaxis plate (Neuro Probe, Inc, Gaithersburg, Md) was precoated with a mixture of type IV collagen (100 ng/25 µL per well) and type I collagen (100 µg/25 µL per well) (Sigma). The collagen-coated plate was either directly used for transmigration assay or further coated with a monolayer of wild-type mouse ECs (1×10⁶ cells per well) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After removal of EC culture media, CD4⁺ T cells or monocytes (25 000 in 25 µL of 1% bovine serum albumin RPMI) were then added to the collagen–EC–precoated or collagen–precoated 96-well chemotaxis plate that contained 30 µL of 1% bovine serum albumin (Sigma) RPMI with or without chemokine SFD1α (0, 10, 100, 1000 ng/mL; PeproTech Inc, Rocky Hill, NJ) in the bottom chamber. After 1.5 hours (T cells), 3 hours (PBMCs), or 12 hours (T cells or PBMCs in collagen–EC–coated plate) of culture, nontranslocated cells on the top transwell were removed, and transmigrated cells in the bottom chambers were precipitated. Cells were transferred into a fluorescent-activated cell sorter tube and fixed with 100 µL of 1% paraformaldehyde, and 5 µL of 15-µm Polybead polystyrene beads was added (Polysciences, Inc, Warrington, Pa) for fluorescent-activated cell sorter analysis. Cell counts were normalized to 2500 beads per sample, and data are presented as fold changes in number of migrating cells compared with wells that received no chemokine treatment (mean of 4 experiments).

**Cysteine Protease Active Site Labeling and MMP Gelatinase Zymogram**

Cysteine protease active site labeling was performed as described previously. Briefly, we incubated cell extract (20 µg) in 100 µL of P.5.5 buffer containing 1 mmol/L EDTA, 40 mmol/L Na acetate, 1% Triton, and 12 mmol/L diithiothreitol with [³⁵S]-JPM at 37°C (1 hour), followed by separation on 12% SDS-PAGE. Gel was stained with Coomassie blue, destained, dried, and exposed to an x-ray film. MMP gelatinase zymography was performed as described. Briefly, an equal amount of protein (7 µg) from each sample was separated on a 10% SDS-PAGE containing 1 mg/mL gelatin. After electrophoresis, the gel was washed twice for a total of 30 minutes in 2.5% Triton, followed by incubation for 18 hours at 37°C in an assay buffer containing 200 mmol/L NaCl and 10 mmol/L CaCl₂ in 40 mmol/L Tris-HCl (pH 7.5). The gel was stained with Coomassie blue and destained. Clear zones of lysis indicated MMP gelatinase activity.

**Cysteine Protease Cathepsin Activity Quantification in PBMCs and CD4⁺ T Cells**

Cat L activity in PBMCs and CD4⁺ T cells was quantified with the fluorogenic InnoZyme Cat L activity kit according to the
maker’s instruction (Calbiochem). PBMCs and CD4+ T cells were lysed in an assay buffer (pH 5.5), and protein concentrations were determined with the BCA kit (Bio-Rad Laboratories, Hercules, Calif). Cell lysates were diluted with buffer (pH 5.5) to a final concentration of 1 mg/mL, and 50 μL per sample was used for the assay with the fluorogenic Cat substrate Z-Phe-Arg-AMC in the presence of the Cat B inhibitor CA074. Purified Cat L was used as the standard for the assay. Data were presented as micromole of active cathepsins in 1 mg/mL cell extract.

In Vitro MMP-9 Degradation by Cat L

Purified mature human neutrophil MMP-9 and recombinant human pro-MMP-9 (0.5 μg/20 μL; Calbiochem) were digested with various amounts of recombinant human Cat L (Calbiochem) (from 250 to 7 nmol/L) in a buffer (pH 5.5) containing 1 mmol/L EDTA, 40 mmol/L Na acetate, 1% Triton X-100, and 12 mmol/L dithiothreitol at 37°C for 1 hour, followed by separation on 12% SDS-PAGE, Coomassie blue staining, and destaining.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Cat L Deficiency Reduces Atherosclerotic Lesion Sizes in LDLr−/− Mice

SMCs, ECs, and macrophages from human atherosclerotic lesions express high levels of Cat L compared with normal human vessels, suggesting its participation in atherogenesis. To assess the role of Cat L in atherogenesis, we introduced Cat L deficiency on the background of LDLr−/− mice, which develop atherosclerotic lesions after consuming a Western diet. LDLr−/− Cat L−/− mice are fertile and consume food and water normally. Although hair loss typical of Cat L−/− mice also was noticed in the LDLr−/− Cat L−/− mice, we did not observe any anatomic abnormality of the hearts or vessels or mortality changes in these mice compared with LDLr−/− mice, although some Cat L−/− mice have been shown to develop cardiomyopathy after 1 year of age.

After 12 weeks on a Western diet, LDLr−/− Cat L−/− and LDLr−/− Cat L+/+ mice showed reduced atherosclerosis (50% [P=0.0006] and 77% [P=0.0002], respectively; Figure 1B) compared with LDLr−/− Cat L+/+ mice. Representative sections immunostained for macrophages are presented in Figure 1A. Additionally, we observed a similar reduction in the intimal area of longitudinal sections of aortic arches compared with LDLr−/− Cat L+/+ mice (P=0.003, P=0.0002, respectively, Mann-Whitney test; Figure 1C). We also found a less marked but still significant reduction in media size in LDLr−/− Cat L−/− mice (30%; P=0.001) compared with LDLr−/− Cat L+/+ mice at this time point (Figure 1D). After 26 weeks on a Western diet, LDLr−/− Cat L−/− mice also had significantly lower lesion grades (23%; P=0.005) and smaller lesion intimal areas (Figure 1B).
leukocyte transendothelial migration requires Cat L. The Cat L gene-dose effect extended to lesion SMC content. Like leukocytes, α-actin–positive SMC levels decreased significantly in LDLr−/−CatL+/mice (46% reduction; \( P=0.003 \)) and decreased further in LDLr−/−Cat L−/−mice (77% reduction; \( P=0.02 \)) relative to SMCs in LDLr−/−Cat L+/+mice after 12 weeks of Western diet (Figure 2C).

Although much more advanced lesions were formed in LDLr−/−Cat L−/−mice after 26 weeks on a Western diet compared with those that consumed 12 weeks of a Western diet (Figure 1A), lesion SMC density did not change significantly, consistent with prior studies. However, like lesion grades presented in Figure 1A, lesion SMC contents in LDLr−/−Cat L−/− and LDLr−/−Cat L+/− mice also caught up at later time point (26 weeks) (Figure 2C). Although differences in macrophage and CD4+ T-cell content among all 3 groups also disappeared after 26 weeks of a Western diet (Figure 2A and 2B), LDLr−/−Cat L−/− and LDLr−/−Cat L+/− mice formed much smaller lipid cores (46% \([P=0.01]\) and 78% \([P=0.007]\), respectively) compared with LDLr−/−Cat L+/+ control mice (Figure 2D), consistent with reduced lesion grade (Figure 1B) or intima area (Figure 1C) in these mice at this time point.

Development of atherosclerosis frequently is associated with lesion cell apoptosis or proliferation. TUNEL assay and Ki67 polyclonal antibody–mediated immunostaining did not reveal significant differences in cell apoptosis or proliferation (data not shown) in mice lacking either 1 Cat L allele (LDLr−/−Cat L−/+ mice) or both Cat L alleles (LDLr−/−Cat L−/− mice), suggesting that Cat L participates in atherosclerosis via mechanisms other than lesion cell survival or growth.

### Cat L Deficiency Reduces Lesion Collagen Content and Medial Elastin Degradation

Cat L is one of the most potent mammalian elastases and collagenases. Therefore, Cat L deficiency likely affects vessel wall collagen content and internal elastic lamina integrity. Consistent with our hypothesis, at both the 12-
26-week time points, vessel wall collagen content grade (Figure 3A), as defined in Figure 3B, and internal elastic lamina degradation grades (Figure 3C), as defined previously, decreased in LDLr/H11002/Cat L/H11002 mice and decreased even more in LDLr/H11002/Cat L/H11002 mice. Decreased collagen levels in LDLr/H11002/Cat L/H11001 and LDLr/H11002/Cat L/H11002 mice (Figure 3A) may result from reduced SMC content (Figure 2C) and increased collagenase activity resulting from the absence of Cat L expression. In contrast, reduced medial elastica degradation may be associated with deficient Cat L expression because high levels of Cat L expression were found in medial SMCs in human atherosclerotic lesions.

Role of Vascular Cell Cat L in Matrix Protein Degradation In Vitro
Elastin and collagen degradation in atherosclerotic lesions correlates with Cat L gene doses. Mice lacking 2 Cat L alleles (LDLr/Cat L) demonstrated more advanced elastin and collagen remodeling than those missing 1 allele (LDLr/Cat L) at both the 12- and 26-week time points (Figure 3A and 3C), suggesting that vascular cell–derived Cat L activity contributes to the progression of atherosclerosis and plaque vulnerability in mice. SMCs, ECs, macrophages, and lymphocytes are important vascular cells that may release Cat L for extracellular matrix remodeling during atherogenesis.
assess the potential role of Cat L in vascular cell collagenolysis and elastolysis, we isolated aortic SMCs and ECs from LDLr−/−Cat L−/− and LDLr−/−Cat L−/− mice and performed elastase and collagenase activity assays under acidic condition in the presence of the cysteine protease cathepsin inhibitor E64d (20 μmol/L) and the Cat L–selective inhibitor CLIK148, which specifically inhibits Cat L activity in concentrations between 2 and 5 μmol/L.8 Unlike macrophages7 and T cells (below), which express amounts of the potent elastases and collagenases Cat S and K high enough to obscure the Cat L elastase/collagenase assay, quiescent SMCs and ECs express only Cat L, not collagenolytic/elastinolytic CatKo orS.11,12 In SMCs, CLIK148 inhibited elastin (Figure 4A) and collagen degradation (Figure 4B) in a dose-dependent fashion from 2 to 20 μmol/L. At 2 to 5 μmol/L, CLIK148 inhibition of elastin and collagen degradation resembled that of LDLr−/−Cat L−/− SMCs (Figure 4A and 4B). In contrast, the reduction in elastin (Figure 4C) and collagen (Figure 4D) degradation in ECs was much weaker with CLIK148 (2 to 5 μmol/L) or in LDLr−/−Cat L−/− ECs, suggesting that Cat L plays a minor role in EC-mediated elastinolysis and collagenolysis. An interesting finding is that higher concentrations of CLIK148 (20 μmol/L) demonstrated significantly reduced EC elastin and collagen degradation comparable to E64d-treated ECs (Figure 4C/D), suggesting that CLIK148 affected cysteine proteases other than Cat L at this concentration.8 Therefore, Cat L derived from SMCs may play a much larger role than Cat L from ECs in extracellular elastin and collagen degradation.

Cat L deficiency in SMCs may alter the expression or activity of other cysteine protease cathepsin family members, which may indirectly explain reduced elastase and collagenase activity in LDLr−/−Cat L−/− SMCs (Figure 4A and 4B). To test this possibility, we labeled SMC lysates from LDLr−/−Cat L−/− and LDLr−/−Cat L−/− mice with [125I]−JPM, which identifies only active cysteine proteases. Labeling of cysteine protease active sites revealed no significant differences in the activity of other cysteine protease cathepsins, including Cat B, H, and C in SMCs from LDLr−/−Cat L−/− and LDLr−/−Cat L−/− mice (Figure 4E, top). SDS-PAGE Coomassie blue staining confirmed equal protein loading (Figure 4E, bottom).

**Cat L Deficiency Attenuates SMC Transmigration Through Elastin Matrix**

A recent study demonstrated cysteine protease Cat S localization on the SMC surface in association with cell surface integrin and suggested that this enzyme may facilitate SMC transmigration through extracellular matrix.21 Cat L may act in a similar fashion. Reduced elastin degradation in vivo in LDLr−/−Cat L−/− mice fed a Western diet for 12 or 26 weeks (Figure 3C) and in vitro in SMCs isolated from the same mice (Figure 4A) supports the participation of SMC Cat L in mediating internal elastica lamina degradation, which leads to impaired SMC accumulation in lesions in LDLr−/−Cat L−/− mice.
mice (Figure 2C). To examine the possible role of Cat L in SMC cross-elastin barrier migration, we performed an in vitro SMC transmigration assay using an elastin matrix– precoated transwell assay. By seeding SMCs on the top of elastin-coated transwells, we detected significant retardation of elastin-mediated transmigration of SMCs from LDLr−/−Cat L−/− mice compared with those from LDLr+/+ mice (Figure 4F), supporting a role of SMC-derived Cat L in medial elastin fragmentation, SMC migration through this biological barrier, and accumulation in the intimal lesions.

### Cat L Deficiency Impairs Monocyte and Lymphocyte Transmigration

In addition to transmigration of medial SMCs into the lesion, atherosclerosis involves infiltration of blood-borne leukocytes into the intima. Leukocyte migration across the endothelium and subjacent basement membrane may involve degradation of matrix proteins, including type I and type IV collagens. At 12 weeks, lesion characterization demonstrated that the absence of Cat L activity significantly reduced macrophage contents in lesions (Figure 2A). Decreased expression of Cat L also reduced lesion macrophage accumulation in a gene-dose–dependent manner (Figure 2B), suggesting that Cat L participates in blood-borne leukocyte infiltration during atherosclerosis development, a hypothesis supported by our in vitro transwell chemotaxis assay. Both PBMCs (Figure 5A) and CD4+ T cells, although less profound (Figure 5B), from LDLr−/−Cat L−/− mice demonstrated significantly reduced transmigration through a collagen type I and IV matrix compared with cells from LDLr+/+ Cat L+/+ mice in this SDF1α-mediated chemotaxis assay, suggesting that Cat L importantly assists leukocyte matrix degradation and translocation from the lumen to the neointima. Furthermore, PBMCs from LDLr−/−Cat L−/− mice also showed significant retardation of transmigration through an artificial vessel wall constructed with a layer of collagen matrix covered with a monolayer of mouse ECs (Figure 5C). In contrast, CD4+ T cells from LDLr−/−Cat L−/− mice did not act significantly differently from those from LDLr+/− mice in transmigration through this artificial vessel wall (not shown), suggesting that additional mechanisms are involved in reducing the number of lesion T cells in LDLr−/−Cat L−/− mice (Figure 2B). Consistent with this hypothesis, lymphocytes from Cat L−/− mice proliferate significantly less than those from Cat L+/+ mice. In the present study, we detected significant fewer lymphocytes in the blood of LDLr−/−Cat L−/− mice than LDLr+/−Cat L+/+ mice (4.1 ± 0.7 versus 7.8 ± 0.7; $P = 0.003$), whereas the numbers of total white blood cells, monocytes, eosinophils, and basophils are no different. Higher numbers of neutrophils in LDLr−/−Cat L−/− mouse blood (8.6 ± 1.6 versus 2.6 ± 0.5; $P = 0.002$) also were observed, which may account for an unchanged total white blood cell content between the 2 types of mice.
Reduced transmigration of LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> leukocytes might not result solely from the lack of Cat L activity but also from impaired expression of other matrix-degrading pro tease. Both cysteine protease cathepsin active site labeling and MMP zymography argue against this hypothesis. The former, with [125I]-JPM, detected no obvious effect of Cat L deficiency on other cathepsin activity in PBMCs. All major cysteine proteases, including Cat B, S, K, and C, which comigrate with Cat L, remained unaffected by Cat L deficiency (Figure 5D). The cysteine protease cathepsin activity profile of CD4<sup>+</sup> T cells differs slightly. Although Cat B activity remained similar in LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> CD4<sup>+</sup> T cells and LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> CD4<sup>+</sup> T cells, the activities of other cathepsins, including Cat S, K, and C, decreased slightly in LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> T cells. In addition, we detected 2 unknown active cathepsins, 1 below the Cat L signal and another above the pre-Cat L band in CD4<sup>+</sup> T cells. Quantitatively, we measured Cat L activity against its substrate Z-Phe-Arg-AMC in PBMCs and CD4<sup>+</sup> T-cell lysate from LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> and LDLr<sup>−/−</sup> mice in the presence of the Cat B–selective inhibitor CA074. Consistent with the data from the cysteine protease active site labeling assay, we observed reduced Z-Phe-Arg-AMC cleavage in PBMCs and CD4<sup>+</sup> T-cell lysates from LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> mice (Figure 5E). The remaining cathepsin activity in LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> mice could be from Cat S, K, and C (Figure 5D and 5E). Even more interesting, a gelatin gel zymogram demonstrated that MMP-9, a 92-kDa gelatinase important for vascular wall remodeling,<sup>23,24</sup> increased in both PBMCs and CD4<sup>+</sup> T cells from LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> mice (Figure 5F) when an equal amount of protein viewed by Coomassie blue–stained SDS-PAGE was used from each cell type (Figure 5G). Increased levels of MMP-9 in PBMCs and CD4<sup>+</sup> T cells from LDLr<sup>−/−</sup> Cat L<sup>−/−</sup> mice suggest that Cat L may inactivate MMP-9 either directly or indirectly, a hypothesis supported by in vitro degradation of recombinant MMP-9 by recombinant Cat L.
Although we do not know whether Cat L degrades MMP-9 in vivo, our in vitro experiment demonstrates that Cat L catabolizes both mature and pro–MMP-9 in a dose-dependent fashion (Figure 5H and 5I). Furthermore, increased expression of MMP-9 in LDLr⁻/⁻ Cat L⁻/⁻ cells in our model suggests that MMP activity does not account for reduced PBMCs and CD4⁺ T-cell transmigration through collagen I/IV matrix (Figure 5A and 5B).

**Discussion**

Elastinolytic cathepsins, including Cat S,⁵ K,²⁵ V,²⁶ and L,⁷ which exhibit high homology among all known papain family members,²⁷ are inflammatory cytokine-inducible cysteine proteases highly expressed in human atherosclerotic lesions,⁴,⁸,²⁶ compatible with an important role in this vascular disorder. Each of these enzymes exerts distinct actions under physiological conditions. For instance, antigen-presenting cells, ie, B cells and dendritic cells, express Cat S preferentially, consistent with the role of this enzyme in major histocompatibility class II–mediated immunity.¹⁴ Although not required for B cells or dendritic cells, Cat L participates crucially in thymic epithelial cell antigen presentation and CD4⁺ T-cell thymic selection.⁹ Cat L–deficient mice have reduced levels of peripheral CD4⁺ T cells but increased CD8⁺ T cells.⁹ Similar to Cat L, thymic epithelial cell antigen presentation requires Cat V. Unlike Cat L, which is widely expressed, thymus and testis selectively express Cat V.²⁸ In contrast, but consistent with its functions in bone resorption,²⁹ Cat K localizes primarily in osteoclasts.²⁵ Cat K deficiency in humans⁹ and mice²⁹ leads to impaired bone growth. However, in inflammatory or pathological conditions, such as atherosclerosis,⁴,²⁶ chronic obstructive pulmonary disease,³¹,³² and malignant tumors,³³ all tested tissues or cells were found to express these elastinolytic cathepsins at much higher levels, suggesting their roles as inflammatory markers or as direct participants in disease pathogenesis. Our prior studies⁵ and those from Lutgens et al²⁷ demonstrated that Cat S and K participate directly in atherogenesis in either LDLr⁻/⁻ mice or ApoE⁻/⁻ mice but with distinct mechanisms. Thus, it is plausible that Cat L and V also participate in atherogenesis with different mechanisms.

Although our understanding of the involvement of Cat L in atherogenesis remains incomplete, we suggest here that Cat L plays at least 2 roles. Similar to the observations from the Cat S–deficient atherosclerotic LDLr⁻/⁻ mice, in which internal elastic laminae were better preserved compared with atherosclerotic LDLr⁻/⁻ mice wild type for Cat S,⁵ LDLr⁻/⁻/Cat L⁻/⁻ and LDLr⁻/⁻ Cat L⁻/⁻ mice also demonstrate reduced elastin degradation compared with LDLr⁻/⁻ Cat L⁺/⁺ mice at both the 12- and 26-week time points (Figure 3C), suggesting that Cat L participates in internal elastic lamina degradation. Indeed, SMCs from LDLr⁻/⁻ Cat L⁻/⁻ mice or those treated with the Cat L-selective inhibitor CLI148 at concentrations that affect only Cat L activity (2 to 5 μmol/L) demonstrated significantly reduced elastinolysis in vitro (Figure 4A). Reduced elastinolysis or better-preserved internal elastica in LDLr⁻/⁻ Cat L⁻/⁻ mice may delay medial SMC migration into the neointima, thus explaining the reduced levels of SMCs in lesions (Figure 2C) and possibly resulting in reduced levels of collagen (Figure 3A). Consistent with this hypothesis, Cat L–deficient SMCs demonstrated delayed transmigration through an elastin layer in vitro (Figure 4F). Cat L–deficient monocytes and lymphocytes demonstrated reduced transmigration through collagen type I– and IV–coated and, even more importantly, an EC monolayer–coated transwell membrane in a chemotaxis assay (Figure 5A through 5C), suggesting an additional role of Cat L in the transmigration of blood-borne leukocytes through the endothelial monolayer and the subendothelial basement membrane. Decreased in vitro transmigration of Cat L–deficient T cells and monocytes through collagen type I and IV mixture–coated transwells may explain reduced macrophage (Figure 2A) and CD4⁺ T-cell (Figure 2B) levels in lesions of LDLr⁻/⁻ Cat L⁻/⁻ mice.

More provocatively and unlike the case with other diseases examined in Cat L–deficient mice, Cat L affects atherosclerotic lesion grade or intima size, lesion content of SMCs and CD4⁺ T cells, and degradation of lesion elastin and collagen in LDLr⁻/⁻ mice in a gene-dose–dependent fashion. For instance, reduced peripheral CD4⁺ T-cell content,⁹ defective thymus stroma cell invariant chain processing,⁹ impaired hair follicle morphogenesis,¹⁷,³⁴ and dilated cardiomyopathy¹⁸ are evident in Cat L⁻/⁻ mice but not in Cat L⁺/⁺ mice. All Cat L⁻/⁻ mice act like Cat L⁺/⁺ mice. These studies did not reveal gene-dose effects, yet Cat L appears to participate in atherogenesis in a gene-dose–dependent manner.

Cysteine proteases likely participate in MMP activation. Inhibition of cysteine protease activity by E64d reduces MMP-9 activity in a rat focal cerebral ischemia model,³⁵ and activation of MMP-1 in interleukin-1β–stimulated gingival fibroblasts requires Cat B.³⁶ However, increased expression of MMP-9, -13, and -14 was detected in osteoclasts from Cat K–deficient mice,³⁷ suggesting that cysteine proteases play dual roles in regulating MMP activity. The present study demonstrated increased MMP-9 activity in PBMCs and CD4⁺ T cells from LDLr⁻/⁻ Cat L⁻/⁻ mice (Figure 5F) and degradation of both mature and pro–MMP-9 in vitro by recombinant Cat L (Figure 5H and 5I), suggesting that cysteine proteases both activate and inactivate MMP. Whether Cat L degrades MMP-9 in vivo remains unknown. However, in vitro chemotaxis transmigration assay suggests that T cell– and monocyte-derived MMP activity plays a minor role in their translocation from the lumen to the neointima. The finding that macrophage infiltration into the neointima remains unaffected in MMP-9–null mice after carotid artery injury supports this hypothesis.³⁸

The present study supports the hypothesis that Cat L participates directly in atherogenesis by mediating SMC-derived degradation of internal elastica, SMC migration and accumulation in the neointima, and transmigration of blood-borne leukocytes into the lesion. The availability of various potent Cat L–selective inhibitors³⁸ suggests novel therapeutic strategies for human atherosclerosis by targeting Cat L activity.⁴⁰

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Disclosures

None.

References

The enzymes cathepsins S, K, and L potently degrade elastin and collagen and may participate in atherogenesis. Human atherosclerotic lesions exhibit elevated activities of these proteases but reduced expression of their endogenous inhibitor cystatin C. In mice, deficiency of cathepsins S and K significantly reduces diet-induced atherosclerosis, perhaps by different mechanisms. In contrast, lack of their endogenous inhibitor greatly enhanced artery wall remodeling and atherosclerosis, indicating a critical role for the balance between proteases and protease inhibitors in maintaining arterial integrity. This study provides direct evidence that cathepsin L participates importantly in atherogenesis. Absence of cathepsin L reduces significantly atherosclerosis in a gene-dose–dependent manner in low-density lipoprotein receptor–deficient mice consuming a Western diet. These data show that cathepsin L influences atherogenesis independently of the related enzymes cathepsins S and K. Inhibition of each of these cathepsins may diminish or delay the development of atherosclerosis. Several cysteine protease cathepsin inhibitors have entered clinical trials for osteoporosis, cancer, and certain autoimmune diseases. Although the complete absence of cathepsin L may cause hair loss and affect peripheral CD4+ T cells and occasional cathepsin L–deficient mice may develop cardiomyopathy, cathepsin L heterozygous mice and those treated with cathepsin L–selective inhibitors have normal hair growth, T-cell development, and organ histology but reduced atherosclerosis. The current observations identify cathepsin L as a novel drug target for atherosclerosis and its complications.
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