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## Cathepsin L Activity Is Essential to Elastase Perfusion–Induced Abdominal Aortic Aneurysms in Mice

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### Abstract

**Objective**—The development of abdominal aortic aneurysms (AAA) requires extensive aortic wall matrix degradation. Human AAA lesions express high levels of cathepsin L (CatL), one of the most potent mammalian elastases. Whether this protease participates directly in AAA pathogenesis, however, is unknown.

**Methods and Results**—We generated experimental AAA with aortic elastase perfusion in mice and established an essential role of CatL in AAA formation. After 14 days postperfusion, most wild-type (Ctsl<sup>+/+</sup>) mice developed AAA, but none of the CatL-deficient (Ctsl<sup>-/-</sup>) mice did. AAA lesion macrophage contents, CD4<sup>+</sup> T cell numbers, CD31<sup>+</sup> and laminin-5 angiogenic fragment  $\gamma$ 2<sup>+</sup> microvessel numbers, and elastin fragmentation were all significantly lower in Ctsl<sup>-/-</sup> mice than in Ctsl<sup>+/+</sup> mice. While lesions from Ctsl<sup>-/-</sup> mice contained fewer Ki67<sup>+</sup> proliferating cells than did Ctsl<sup>+/+</sup> mice, the absence of CatL did not affect lesion apoptotic cell contents or medial smooth-muscle cell loss significantly. Mechanistic studies indicated that the absence of CatL reduced lesion chemokine monocyte chemoattractant protein-1 content, macrophage and T-cell in vitro transmigration, and angiogenesis, and altered the expression and activities of matrix metalloproteinases and other cysteinyl cathepsins in inflammatory cells, vascular cells, and AAA lesions.

**Conclusion**—CatL contributes to AAA formation by promoting lesion inflammatory cell accumulation, angiogenesis, and protease expression.

### Keywords

aneurysms; angiogenesis; macrophages; cathepsin L; elastase

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The development of abdominal aortic aneurysms (AAA) involves extensive aortic wall remodeling, in which proteases play an essential role.<sup>1</sup> Matrix metalloproteinases (MMP) and serine proteases may participate directly in experimental AAA in animals.<sup>2–5</sup> Most of the cysteine proteases called cathepsins are regulatory proteases that are expressed in

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Disclosures  
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restricted tissues under physiological conditions but are induced in almost all tested tissues or cells by pathological stimuli, particularly by inflammatory cytokines.<sup>6</sup> Human AAA lesions are rich in inflammatory cells, including T cells, macrophages, neutrophils, and mast cells.<sup>7,8</sup> All of these cells produce inflammatory cytokines that enhance vascular cell cathepsin expression.<sup>6,9</sup> Cathepsins (Cat) S, L, and K are potent elastases that are highly expressed in human AAA lesions,<sup>6,10-12</sup> suggesting their participation in AAA. Indeed, mice lacking CatC are resistant to aortic elastase perfusion-induced AAA,<sup>13</sup> and the absence of CatK also protected mice from AAA in the same experimental AAA model.<sup>14</sup> Like CatK, CatL is a potent mammalian elastase that is localized to smooth-muscle cells (SMC), endothelial cells (EC), and macrophages in human AAA lesions.<sup>6,10</sup> We showed previously that CatL contributed to atherogenesis in LDL receptor-deficient (*Ldlr*<sup>-/-</sup>) mice by promoting aortic wall elastin degradation, intima inflammatory cell accumulation, and inflammatory cell protease expression. The absence of CatL reduced atherosclerosis in *Ldlr*<sup>-/-</sup> mice in a gene dose-dependent manner.<sup>15</sup> AAA is clinically different but shares many pathophysiologies with atherosclerosis, including inflammatory cell recruitment, extracellular matrix protein degradation, angiogenesis, and apoptosis.<sup>7,16,17</sup> Given our demonstrated observations of CatL functions in atherosclerosis and the close association between AAA and atherosclerosis, we hypothesized that increased CatL expression in human AAA lesions is not just secondary to the disease but also participates directly in its pathogenesis. This study used CatL-deficient mice and elastase perfusion-induced experimental AAA to test this hypothesis.

## Materials and Methods

### Mouse AAA Model and Lesion Characterization

Ten-week-old male CatL-deficient (*Ctsl*<sup>-/-</sup>) mice<sup>18</sup> and male wild-type (*Ctsl*<sup>+/+</sup>) littermates (all in C57BL/6/129S background) underwent aortic elastase perfusion-induced experimental AAA.<sup>2</sup> Mouse aortic diameters were measured using a surgical microscope (Zeiss Stemi. SV11) equipped with a micrometer eyepiece (14 mm/0.1, SG02.T0218c, Motic Instruments, Inc, Vancouver, Canada), which allowed us to read the aortic diameters under physiological blood pressure at any time during the surgical procedure or during tissue harvesting.<sup>2,13</sup> Mouse aortic diameters were measured and recorded before elastase perfusion and 5 minutes after perfusion restoration. Mouse abdominal aortas were collected at 7 days and 14 days postperfusion. Before mice were euthanized, abdominal aorta diameters were measured and recorded at both the 7-day and 14-day time points. Aortic diameter expansion  $\geq 100\%$  of that before elastase perfusion defined AAA.<sup>2</sup> Each mouse aorta was isolated for both frozen section preparation and tissue protein extraction in a pH 5.5 buffer containing 1% Triton X-100, 40 mmol/L sodium acetate, and 1 mmol/L EDTA.<sup>19</sup> Frozen sections were used for immunostaining for macrophages (Mac-3), SMC ( $\alpha$ -actin), T cells (CD4), monocyte chemoattractant protein-1 (MCP-1), EC (CD31), elastin (Verhoeff-van Gieson), apoptotic cells (TUNEL), and proliferating cells (Ki67), as described previously.<sup>9,19,20</sup> Corresponding purified IgG isotypes were used routinely as immunostaining negative controls. Elastin fragmentation and SMC content in media were graded with the same keys, as we described recently.<sup>20</sup> Numbers of CD4<sup>+</sup> T cells, MCP-1<sup>+</sup> cells, TUNEL-positive apoptotic cells, Ki67<sup>+</sup> proliferating cells, and CD31<sup>+</sup> or lamin-5  $\gamma 2$ <sup>+</sup> microvessels in AAA lesions were counted blindly by 2 independent observers. Mac-3<sup>+</sup> macrophage areas in AAA lesions were measured with computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD).

To produce AAA by periaortic chemical injury, 8-week-old male *Ctsl*<sup>+/+</sup> and *Ctsl*<sup>-/-</sup> mice were used for aorta CaCl<sub>2</sub> (0.25 mol/L) injury as described.<sup>3</sup> Aortic diameters were measured before CaCl<sub>2</sub> application and 4 weeks after the injury using the same micrometer eyepiece.

### Real-Time Polymerase Chain Reaction

Mouse aortic SMC and mouse heart microvessel endothelial cells (MHEC) were prepared as described elsewhere.<sup>19,21</sup> Total cellular RNA was extracted from SMC and MHEC using a TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA samples were then treated with a RNase-free DNase (Ambion, Carlsbad, Cal) to remove genomic DNA contaminants. Equal amounts of RNA were reverse transcribed, and quantitative PCR was performed in a single-color real-time polymerase chain reaction (RT-PCR) detection system (Stratagene, La Jolla, CA). The mRNA levels of CatS, CatK, CatB, MMP-2, and MMP-9 were normalized to those of housekeeping gene  $\beta$ -actin.

### MHEC Cysteine Protease Active Site Labeling and Gelatin Gel In Situ Zymogram

MHEC were lysed into a pH 5.5 buffer.<sup>19</sup> Five micrograms of protein from each sample were incubated with 12 mmol/L dithiothreitol and 1  $\mu$ L of biotin-conjugated JPM in 30  $\mu$ L of a pH 5.5 buffer for 1 hour at 37°C. Protein samples were then separated on a 12% SDS-PAGE, followed by immunoblot detection with horseradish peroxidase-conjugated avidin.<sup>4</sup> MMP activity was detected with a gelatin gel zymogram, essentially the same as reported previously.<sup>22</sup> Equal protein loading (20  $\mu$ g protein/ lane) was confirmed by immunoblot analysis with goat anti-mouse GAPDH polyclonal antibodies (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA).

### Aortic Ring Assay

An aortic ring assay was used to test the role of CatL in angiogenesis. In brief, a 96-well plate was coated with 50  $\mu$ L of Matrigel (BD Biosciences, San Diego, CA). A 1-mm long mouse aortic ring from Cts1<sup>+/+</sup> mouse or Cts1<sup>-/-</sup> mouse was laid on top of the solidified Matrigel and covered with 100  $\mu$ L of Matrigel. After solidification at room temperature, 150  $\mu$ L of RPMI (with 10% FBS) was added to each well. After 7 to 10 days of culture, the aortas were photographed, and the endothelial outgrowth was analyzed using Image-Pro Plus software and presented as square millimeters. Basic fibroblast growth factor (bFGF) (10 ng/mL; PeproTech, Rocky Hill, NJ) was used as a positive control.

### In Situ Elastin Zymography

AAA lesion elastinolytic activity was determined in 8- $\mu$ M frozen sections using elastin conjugated with quenched fluorescein (DQ elastin; Invitrogen) as a substrate, which requires cleavage by elastinolytic enzymes to become fluorescent. In brief, DQ elastin (1 mg/mL in H<sub>2</sub>O) was mixed 1:10 with 1% low-melting agarose (Sigma, St. Louis, MO). This mixture (20  $\mu$ L) was added on top of each section, coverslipped, and gelled at 4°C. Following incubation at 37°C (48 hours), fluorescence was examined under fluorescent microscopy. Cysteine protease activity was determined using an EDTA-containing pH 5.5 buffer with or without 20  $\mu$ mol/L E64 days.<sup>9</sup> Zymographic images were acquired using identical shutter conditions. The percentage of fluorescence intensity of each cross section, excluding the media area due to medial elastin filament autofluorescence, was measured using computer-assisted image quantification (Image-Pro Plus software).

### Cell Proliferation Assay

CD4<sup>+</sup> T cell and monocyte proliferation was assessed with the Cell Titer 96AQ Assay kit, according to the manufacturer's instructions (Promega, Madison, WI). T cells and monocytes were plated with serial 2-fold dilutions on 96-well plates starting from 2 $\times$ 10<sup>5</sup> cells in 100  $\mu$ L of 10% FBS RPMI 1640 per well and cultured 2 days at 37°C. Mouse anti-CD3 monoclonal antibody (1  $\mu$ g/mL, Pharmingen, San Diego, CA) was used to coat the 96-well plate for T-cell proliferation assay, as described.<sup>23</sup> Then 20  $\mu$ L of a mixture of

tetrazolium compound and phenazine methosulfate was added, and the absorbance was determined at 492 nm.

### Statistical Analysis

Because of the relatively small sample sizes and data distribution abnormality, we selected the nonparametric Mann-Whitney U test for nonpaired data sets and the Wilcoxon signed-rank test for paired data sets to examine statistical significance throughout this study. Two-way repeated-measures ANOVA test followed by Bonferroni posthoc correction was used for multiple group comparisons.  $P < 0.05$  was considered statistically significant.

## Results

### CatL Deficiency Protected Mice From Elastase Perfusion-Induced AAA

Both  $Ctsl^{-/-}$  and  $Ctsl^{+/+}$  littermates underwent aortic elastase perfusion.<sup>2,18</sup> For unknown reasons, baseline aortic diameters of  $Ctsl^{-/-}$  mice were larger than those of  $Ctsl^{+/+}$  littermates from both the 7-day ( $0.56 \pm 0.01$  versus  $0.50 \pm 0.01$  mm,  $P < 0.05$ ) and 14-day ( $0.54 \pm 0.01$  versus  $0.50 \pm 0.01$  mm,  $P > 0.05$ ) experimental groups, although all mice were 10-week-old males. In mice used for the 7-day time point, at the time of harvesting (day 7), there was no difference between aortic diameters of  $Ctsl^{-/-}$  mice and  $Ctsl^{+/+}$  mice, nor between immediate postperfusion and 7 days postperfusion in  $Ctsl^{-/-}$  mice (Figure 1A). At this time point, no mice from either group developed AAA by definition.<sup>2</sup> In mice used for the 14-day time point, aortic diameters of  $Ctsl^{+/+}$  mice were significantly enlarged at 14 days postperfusion compared with those from  $Ctsl^{-/-}$  mice ( $P < 0.001$ ) or those immediate postperfusion from the same group of mice ( $P < 0.001$ ) (Figure 1B). At this time point, 9 of 11  $Ctsl^{+/+}$  mice developed AAA, as defined by  $>100\%$  of aortic diameter increase, but none of the  $Ctsl^{-/-}$  mice ( $n=11$ ) did.

### CatL Deficiency Reduced AAA Lesion Inflammatory Cell Accumulation

Inflammatory cell infiltration to AAA lesions is an important signature of inflammation in this disease. As in atherosclerotic lesions,<sup>15</sup> the absence of CatL reduced Mac-3<sup>+</sup> macrophage and CD4<sup>+</sup> T-cell accumulation significantly in AAA lesions (Figure 2A/B). Reduced macrophages and CD4<sup>+</sup> T cells in  $Ctsl^{-/-}$  mouse AAA lesions suggest a role of CatL in leukocyte recruitment or proliferation. In an in vitro transwell migration assay, monocytes or CD4<sup>+</sup> T cells from  $Ctsl^{-/-}$  mice transmigrated through collagen-coated or EC monolayer-coated transwells much more slowly than did those from  $Ctsl^{+/+}$  mice.<sup>15</sup> In AAA lesions, we also detected fewer chemokine MCP-1–positive cells in  $Ctsl^{-/-}$  mice than in  $Ctsl^{+/+}$  mice at 14 days postperfusion (Figure 2C). We did not, however, detect significant differences in monocyte or T-cell proliferation between the genotypes (data not shown). These observations suggest that reduced macrophages and CD4<sup>+</sup> T cells in  $Ctsl^{-/-}$  mouse AAA lesions were caused by impaired inflammatory cell recruitment but not by proliferation.

### Impaired Angiogenesis in AAA Lesions From $Ctsl^{-/-}$ Mice

We have shown previously that CatS plays an important role in angiogenesis by degrading antiangiogenic matrix collagen fragments and generating proangiogenic laminin-5 fragments.<sup>24</sup> AAA lesions from  $Ctsl^{-/-}$  mice contained significantly reduced numbers of CD31<sup>+</sup> microvessels at 14 days postperfusion and of proangiogenic laminin-5  $\gamma 2^+$  vessels at both 7 days and 14 days postperfusion, compared with  $Ctsl^{+/+}$  mice (Figure 3A/B). In vitro aortic ring angiogenesis assay showed defects of microvessels sprouting from aortic rings from  $Ctsl^{-/-}$  mice with or without angiogenic factor bFGF (Figure 3C). To assess whether the absence of CatL affected the expression or activities of other proteases, we performed

RT-PCR in MHEC from *Ctsl*<sup>-/-</sup> and *Ctsl*<sup>+/+</sup> mice and demonstrated that the absence of CatL reduced CatB, CatK, and MMP-2 mRNA levels in MHEC (Figure 3D). CysteinyI cathepsin active site labeling with JPM (Figure 3E) and gelatin gel zymogram (Figure 3F) revealed reduced activities of major cathepsins (cathepsins B, L, K, and S) and MMPs (MMP-1, -2, -3, and -9), suggesting that reduced angiogenesis in *Ctsl*<sup>-/-</sup> mice was caused in part by reduced EC protease expression and activities.

### Absence of CatL Did Not Affect AAA Lesion Cell Apoptosis

Although we detected fewer Ki67<sup>+</sup> proliferating cells in AAA lesions from *Ctsl*<sup>-/-</sup> mice than in that of *Ctsl*<sup>+/+</sup> mice (Figure 4A), the absence of CatL did not affect lesion cell apoptosis (Figure 4B), which causes loss of SMC in AAA.<sup>17</sup> Consistent with this observation, medial SMC loss was not different between the 2 genotypes (Figure 4C). These observations suggested that CatL played a negligible role in SMC apoptosis. We tested this hypothesis in cultured aortic SMC. SMC undergo apoptosis after stimulation with antioxidant pyrrolidine dithiocarbamate (PDTC),<sup>25</sup> but SMC from *Ctsl*<sup>-/-</sup> mice showed no significant differences in apoptosis from those of *Ctsl*<sup>+/+</sup> mice (data not shown).

### Essential Role of CatL in Medial Elastin Degradation

As one of the most potent elastases, CatL may be essential to AAA, in which medial elastin degradation is a major molecular mechanism of pathogenesis. As we anticipated, aortic medial elastin fragmentation at the site of AAA development was significantly impaired in *Ctsl*<sup>-/-</sup> mice as compared with *Ctsl*<sup>+/+</sup> mice (Figure 5A). We have shown previously that medial elastin degradation was also impaired in *Ctsl*<sup>-/-</sup> and *Ctsl*<sup>±</sup> mice in the background of *Ldlr*<sup>-/-</sup> after 12 weeks or 26 weeks on an atherogenic diet.<sup>15</sup> SMC from *Ctsl*<sup>-/-</sup> mice had greatly reduced elastolytic activities.<sup>14</sup> RT-PCR demonstrated that the absence of CatL reduced the mRNA levels of CatS, CatK, MMP-2, and MMP-9 in SMC from *Ctsl*<sup>-/-</sup> mice compared with those in *Ctsl*<sup>+/+</sup> mice (Figure 5B), although the reduction of CatS in *Ctsl*<sup>-/-</sup> SMC did not reach statistical significance. Insignificance in CatS expression was likely due to its low expression levels and associated low experimental sensitivity (Figure 5B).

Reduced cysteine protease cathepsin expression and activities in SMC (Figure 5B), MHEC (Figure 3D/3E), T cells, and monocytes<sup>15</sup> in the genetic absence of CatL strongly suggests that CatL participates in regulating other cathepsin expression and activities. Impaired AAA formation in *Ctsl*<sup>-/-</sup> mice may associate with impaired overall protease expressions. To examine this hypothesis, we performed in situ cathepsin elastase zymogram assay. As we showed previously,<sup>4,20</sup> AAA lesions from wild-type mice at 14 days postperfusion contained high levels of elastolytic activities under acidic (pH5.5) conditions that were optimized for cysteinyI cathepsins (fluorescent activities in the adventitia region, Figure 5C, top left panel). Such elastolytic cathepsin activities can be largely inhibited by 20 μmol/L of E64d (Figure 5C, top right panel). In contrast, AAA lesions from *Ctsl*<sup>-/-</sup> mice had greatly reduced adventitia fluorescence (Figure 5C, bottom left panel), and E64d further reduced adventitia elastolytic cathepsin activities (Figure 5C, bottom right panel).

Reduced elastin degradation in the media (Figure 5A) in AAA lesions from *Ctsl*<sup>-/-</sup> mice, impaired expression of cathepsins in aortic SMC from *Ctsl*<sup>-/-</sup> mice (Figure 5B), and decreased elastolytic cathepsin activities in the adventitia (Figure 5C) and possibly in the media from *Ctsl*<sup>-/-</sup> mouse AAA lesions all suggest that the absence of CatL affected medial elastolytic cathepsin expression. To examine this hypothesis, we immunostained AAA lesions from both *Ctsl*<sup>+/+</sup> and *Ctsl*<sup>-/-</sup> mice from the 14-day time point for CatS and CatK. Medial expressions of both tested cathepsins were much lower in AAA lesions from *Ctsl*<sup>-/-</sup> mice than in those from *Ctsl*<sup>+/+</sup> mice (Figure 5D). Reduced medial cathepsin expression

therefore may account in part for better preservation of elastin fragmentation (Figure 5A), and for impaired aorta expansion (Figure 1B), in *Ctsl*<sup>-/-</sup> mice compared with *Ctsl*<sup>+/+</sup> mice.

Intranasal exposure of porcine pancreatic elastase develops secondary immune responses via infiltration of inflammatory cells (eg, macrophages, neutrophils, CD4<sup>+</sup> and CD8<sup>+</sup> T cells) and production of proinflammatory cytokines (eg, IL1 $\beta$ , IL6, TNF- $\alpha$ ) in mouse lungs.<sup>25</sup> The same elastase perfusion in aortas also may have developed these immune responses that were independent from CatL activities, and therefore may have obscured the effect of CatL in AAA formation and progression. To exclude these possibilities and further confirm a role of CatL in AAA, we applied both *Ctsl*<sup>+/+</sup> and *Ctsl*<sup>-/-</sup> mice to a periaortic CaCl<sub>2</sub> injury-induced experimental AAA model. One month after the injury, aortic diameters in *Ctsl*<sup>+/+</sup> mice increased by nearly 50%, whereas those in *Ctsl*<sup>-/-</sup> mice showed negligible changes ( $\approx$ 4%) (Figure 6A). Medial elastin degradation also was reduced significantly in *Ctsl*<sup>-/-</sup> mice compared with *Ctsl*<sup>+/+</sup> mice (Figure 6B), supporting a direct role of CatL in AAA formation.

## Discussion

This study demonstrated an important role of elastolytic CatL in AAA. Although other mechanisms require further investigation, we found that CatL contributed to AAA formation by regulating monocyte and T-cell recruitment, vascular wall matrix protein degradation, lesion cell proliferation, protease expression, and angiogenesis, but it had no effect on SMC or total lesion cell apoptosis. This study also revealed functional differences between CatL and CatK: In human AAA lesions, CatL is expressed mainly in macrophages and weakly in SMC.<sup>10</sup> CatK may have different cellular expression profiles in AAA lesions. For example, CatK is highly expressed in both macrophages and SMC in human atheroma.<sup>27</sup> CatK in human AAA lesions may have similar expression patterns to those in human atherosclerotic lesions. Further, both CatL and CatK are cysteine proteases with potent elastolytic, collagenolytic, and fibrinogenolytic activities,<sup>6,28,29</sup> and even share >50% cDNA sequence homologies,<sup>30</sup> but they may have different activities on other untested substrates. In a separate study, we showed that mice lacking CatK also failed to develop AAA in the same experimental model as the current study, but with different mechanisms.<sup>14</sup> First, although the T-cell number, but not the macrophage content, was significantly lower in AAA lesions from *Ctsk*<sup>-/-</sup> mice than in those from *Ctsk*<sup>+/+</sup> mice, T cells or monocytes from *Ctsk*<sup>-/-</sup> mice showed no differences from those of *Ctsk*<sup>+/+</sup> mice in transmigration through collagen-coated transwells. The absence of CatK affected T-cell proliferation but not monocytes. In contrast, deficiency of CatL did not affect T-cell or monocyte proliferation (data not shown) but significantly reduced their transmigration through collagen-coated or collagen and EC monolayer double-coated transwells,<sup>15</sup> which may explain reduced T cells and macrophages in AAA lesions from *Ctsl*<sup>-/-</sup> mice (Figure 2A/2B). Further, CatL, but not CatK is involved in antigen presentation. Deficiency of CatL reduces circulating CD4<sup>+</sup> T cells,<sup>18</sup> which may also contribute to low CD4<sup>+</sup> T cells in AAA lesions from *Ctsl*<sup>-/-</sup> mice (Figure 2B). Thus, CatK and CatL contributed to AAA lesion inflammatory cell accumulation with different mechanisms. Second, both in vitro aortic ring assay and anti-mouse CD31 monoclonal antibody immunostaining of AAA lesion sections showed that the absence of CatK did not affect microvessel sprouting from aortic rings or CD31<sup>+</sup> microvessel numbers in AAA lesions. In contrast, we hardly detected any microvessel sprouting in aortic rings from *Ctsl*<sup>-/-</sup> mice with or without bFGF (Figure 3C). Furthermore, CD31<sup>+</sup> or laminin-5  $\gamma$ 2<sup>+</sup> microvessel numbers were significantly fewer in *Ctsl*<sup>-/-</sup> mouse AAA lesions than in *Ctsl*<sup>+/+</sup> mouse AAA lesions (Figure 3A/3B). Our studies therefore demonstrated that CatL, but not CatK, affected angiogenesis. We currently do not know the molecular explanation for these observations. Different cathepsins may produce different proangiogenic or antiangiogenic fragments from the matrix; for example, we demonstrated that CatS produces proangiogenic

$\gamma$ 2 fragments from laminin-5.<sup>24</sup> In contrast, CatV—but not CatL, CatB, or CatK—produces antiangiogenic angiostatin-like fragments from plasminogen.<sup>31</sup> CatL and CatK may produce different bioactive molecules from the matrix, a hypothesis that merits further investigation. Third, CatL played negligible roles in SMC apoptosis. PDTC-induced apoptosis of aortic SMC in vitro from *Ctsl*<sup>-/-</sup> mice showed no difference from those of *Ctsl*<sup>+/+</sup> mice. AAA lesion apoptotic cell numbers from *Ctsl*<sup>-/-</sup> mice also were not different from those in *Ctsl*<sup>+/+</sup> mice (Figure 4B). In contrast, CatK is essential to SMC apoptosis and to AAA lesion cell apoptosis. Aortic SMC from *Ctsk*<sup>-/-</sup> mice were very well protected from PDTC-induced apoptosis. TUNEL-positive cells in AAA lesions from *Ctsk*<sup>-/-</sup> mice were much fewer than in those from *Ctsk*<sup>+/+</sup> mice. Therefore, CatK, but not CatL, plays an important role in cell apoptosis. Similar findings have been demonstrated in osteoclasts; the absence of CatK leads to overgrowth of osteoclasts in vitro and in vivo.<sup>32</sup> The role of CatL in cell apoptosis, however, seems much more complicated. Much of the data from CatL genetically deficient or antisense cDNA inactivated cells suggested a protective role of CatL in cell apoptosis.<sup>33</sup> In contrast, CatL inhibition or depletion promoted cell apoptosis in different cell types.<sup>34–36</sup> Therefore, the role of CatL in apoptosis remains controversial and may be cell type-dependent.

In contrast to our unpublished data regarding CatK in mouse experimental AAA, Bai et al recently reported different observations in angiotensin-II (Ang-II) infusion-induced AAA from apolipoprotein E-deficient (*ApoE*<sup>-/-</sup>) mice. In these mice, the presence or absence of CatK did not affect AAA formation.<sup>37</sup> Through unknown mechanisms, Ang-II infusion enhanced peripheral active CD4<sup>+</sup>CD25<sup>+</sup> T cells and Lg6G<sup>+</sup> leukocytes in *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice and increased AAA lesional CD45<sup>+</sup> leukocytes and Mac-3<sup>+</sup> macrophages in these mice.<sup>37</sup> Ang-II-induced increase of inflammatory cells in *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice in both peripheral and AAA lesions may have compensated the effect of CatK deficiency, thereby obscuring the difference in AAA formation between *ApoE*<sup>-/-</sup>*Ctsk*<sup>+/+</sup> and *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice. We examined this hypothesis by infusing Ang-II (1000 ng/min/kg) to *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice. Ang-II infusion did not produce AAA in mice that were wild-type for ApoE, but it increased blood Ly6G<sup>+</sup> neutrophils and Ly6G<sup>+</sup>CD11b<sup>+</sup> activated neutrophils in *Ctsk*<sup>+/+</sup> mice and minimized the differences in blood CD4<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>+</sup> T cells between *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice. Different experimental models therefore may yield different conclusions, although we have not tested whether CatL deficiency also will reduce Ang-II-induced AAA as it did aortic elastase perfusion-induced AAA (Figure 1B) or periaortic CaCl<sub>2</sub> injury-induced AAA (Figure 6A).

CatL is a lysosomal cysteine protease that presumably resides in the late endosomes and lysosomes, which provide an acidic environment for optimal CatL activities.<sup>6</sup> Our longstanding interest has been in how this intracellular protease degrades extracellular elastin in the aortic wall and leads to AAA expansion. Although we did not examine it in this study, seminal studies from Stephen J. Weiss and colleagues demonstrated by culturing human monocyte-derived macrophages with water-insoluble elastin fibers, that macrophages bind tightly onto extracellular elastin, followed by increased expression of macrophage vacuolar-type H<sup>+</sup>-ATPase and rapid acidification in the cell–elastin interface—thus forming sequestered and acidic environments into which CatL and other cathepsins (eg, CatS and CatK) are released from macrophages and deposited into this milieu.<sup>38,39</sup> Aortic SMC may act in the same fashion as macrophages do during elastin lamina fragmentation, and this hypothesis merits further investigation. Another unsolved puzzle is that deficiency of one protease may affect the expression of others. Although absence of CatS did not affect cathepsin and MMP expression or activities in EC,<sup>40</sup> CatL deficiency greatly reduced the expressions of most cathepsins and MMPs in MHEC (Figure 3D) and SMC (Figure 5B). We reported similar observations in chymase- and tryptase-deficient mast cells and monocytes.<sup>4,41</sup> Absence of these serine proteases reduced significantly most cysteinyl

cathepsins or MMPs in these inflammatory cells, although the exact mechanisms involved in these protease expression regulation remain unknown.

Together, our data from experimental AAA establish a selective and important role of CatL in mouse AAA formation. We hope that selective inhibition of CatL using pharmacological inhibitors will benefit patients with AAA, atherosclerosis, and other cardiovascular diseases. Validated biomarkers associated with these potential therapies are essential to assessing the efficacy of selective cathepsin inhibitors. The recent development of potent, highly selective, irreversible or reversible CatL inhibitors<sup>42–46</sup> has allowed examination of the potential efficacy of these small molecule compounds in patients with AAA, or in experimental AAA models. Several serum biomarker candidates, such as peroxiredoxin-1,<sup>46</sup> elastin peptide and plasmin-antiplasmin complex,<sup>47</sup> D-dimer,<sup>48</sup> and mast cell tryptase,<sup>41</sup> have been examined in AAA patients and have correlated with AAA lesion sizes or growth rate. Selective inhibition of CatL may associate with changes in these biomarker candidates. Combination of these biomarker studies and efficacy tests of selective CatL inhibitors in humans and in experimental models, therefore, will be of common interest to the fields of cysteine protease cathepsins and cardiovascular diseases.

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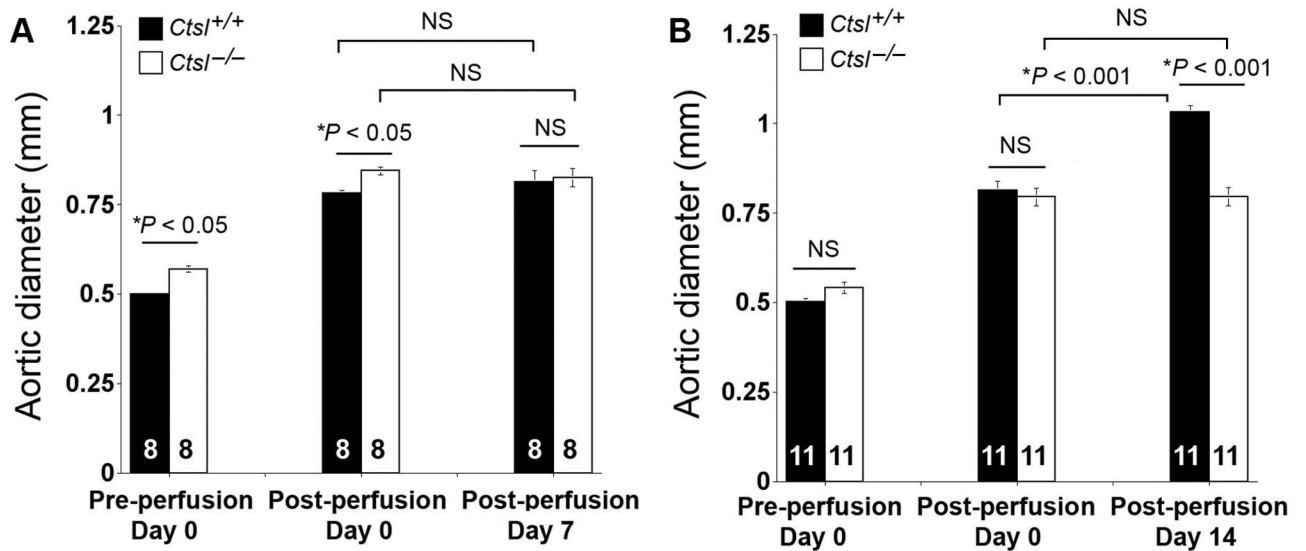
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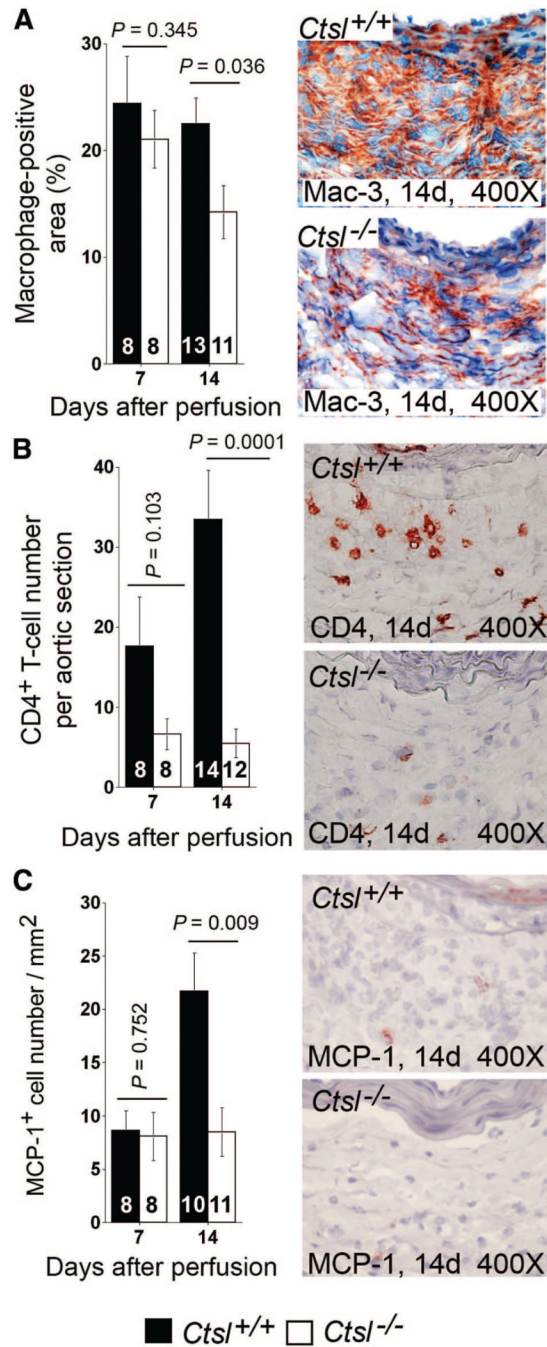
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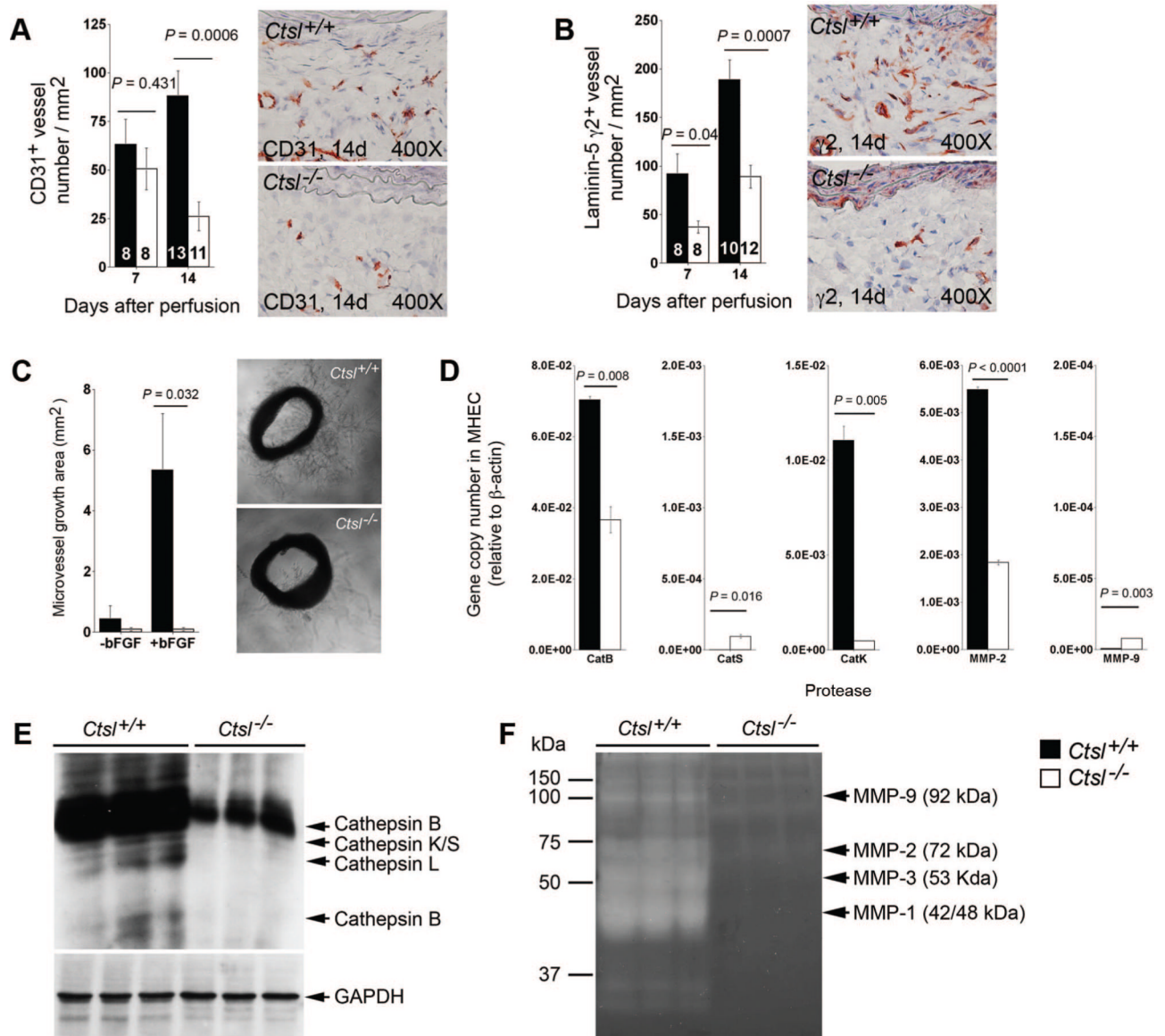


**Figure 1.**

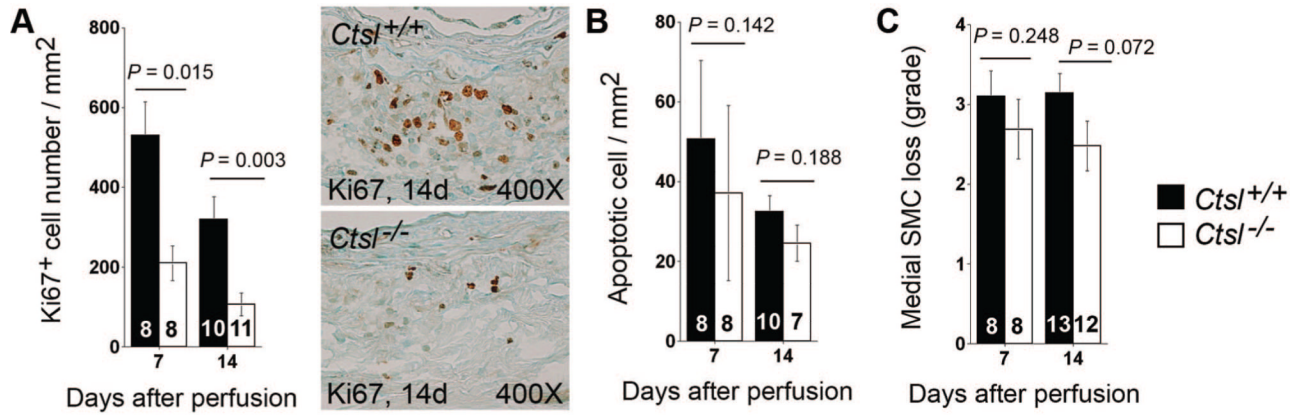
Aortic expansion in elastase perfusion-induced experimental abdominal aortic aneurysms. Aortic diameters from Ctsl<sup>+/+</sup> mice and Ctsl<sup>-/-</sup> mice at different time points: preperfusion, immediate postperfusion (day 0), and at harvesting on day 7 (A) and day 14 (B). Data are mean±SE. The number of mice per group is indicated in each bar.  $P < 0.05$  was considered statistically significant, 2-way repeated-measures ANOVA test followed by Bonferroni posthoc correction. NS indicates not significant.

**Figure 2.**

Abdominal aortic aneurysms (AAA) lesion inflammatory cell accumulation. Mac-3<sup>+</sup> macrophages (A) and CD4<sup>+</sup> T cells (B) accumulated less in AAA lesions from *Ctsl*<sup>-/-</sup> mice than in those of *Ctsl*<sup>+/+</sup> mice at 14 days postperfusion. At the same time point, MCP-1<sup>+</sup> cell numbers were also fewer in AAA lesions from *Ctsl*<sup>-/-</sup> mice than in *Ctsl*<sup>+/+</sup> mice (C). Data are mean±SE. The number of mice per group is indicated in each bar. *P*<0.05 was considered statistically significant; Mann-Whitney U test. Representative images are shown to the right in each panel. All measurements are from the entire lesion including both adventitia and media.

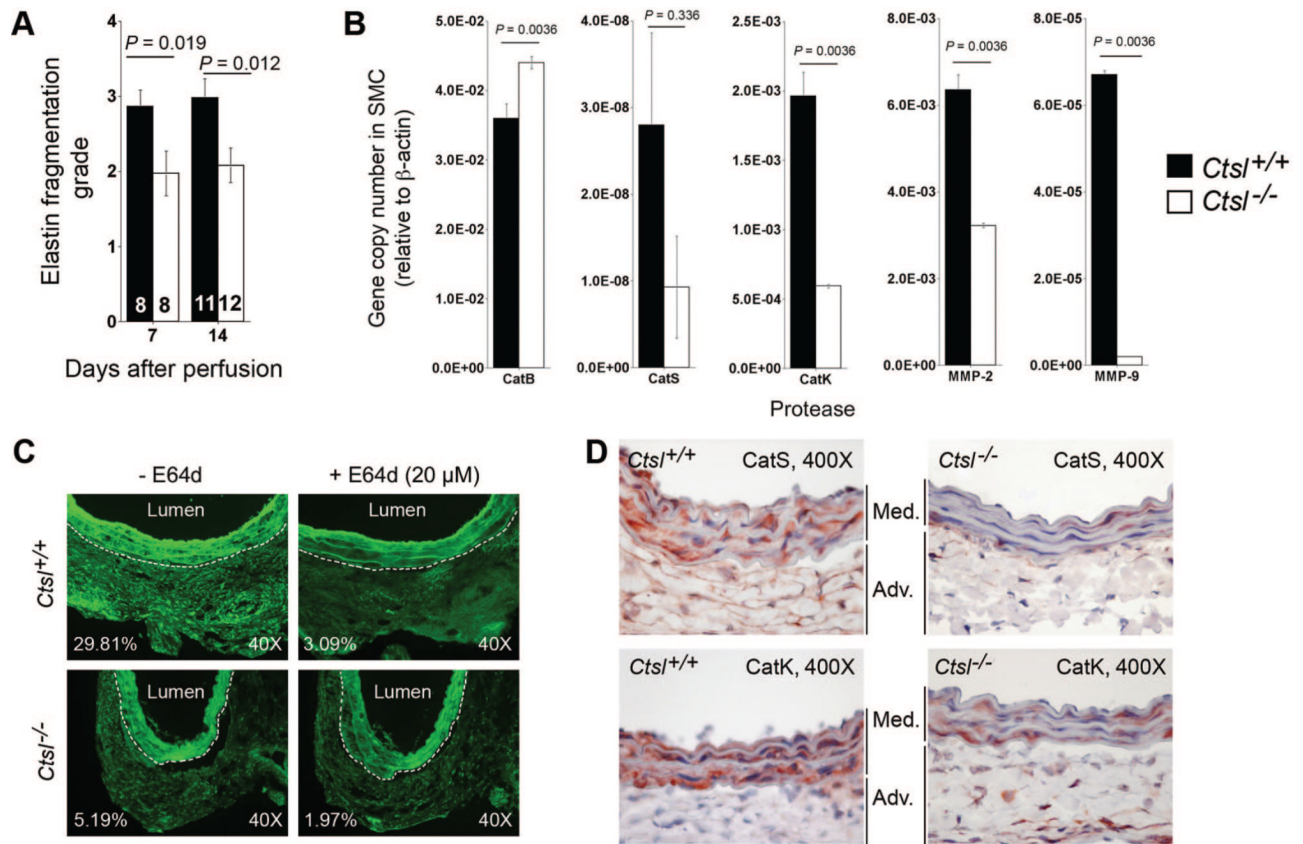


**Figure 3.** Cathepsin L function in neovascularization in abdominal aortic aneurysms (AAA) lesions. CD31<sup>+</sup> (A) and proangiogenic laminin-5 fragment  $\gamma 2^+$  (B) microvessel numbers were reduced in AAA lesions from *Ctsl*<sup>-/-</sup> mice. The number of mice per group is indicated in each bar. Both measurements are from the entire lesion including adventitia and media. Aortic ring assay in vitro demonstrated impaired microvessel sprouting from *Ctsl*<sup>-/-</sup> mouse aortic rings with or without angiogenic factor bFGF (C). Representative images for panels A–C are shown to the right. RT-PCR showed reduced transcripts of cathepsins B and K, and matrix metalloproteinases (MMP)-2 in microvessel endothelial cells (MHEC) from *Ctsl*<sup>-/-</sup> mice compared with those from *Ctsl*<sup>+/+</sup> mice (D). Cysteine protease active site labeling with JPM (E) and gelatin gel zymogram (F) demonstrated impaired cathepsin and MMP activities in MHEC from *Ctsl*<sup>-/-</sup> mice, respectively. GAPDH immunoblot was used for protein loading control. All data are mean $\pm$ SE.  $P < 0.05$  was considered statistically significant; Mann-Whitney U test.



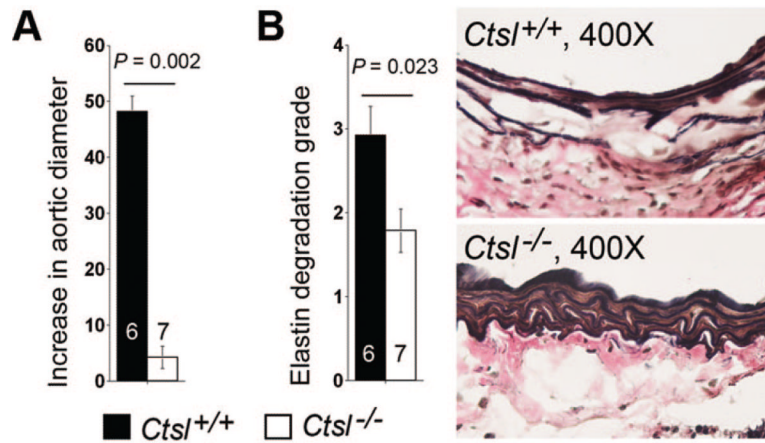
**Figure 4.**

Cathepsin L deficiency and abdominal aortic aneurysms (AAA) lesion cell proliferation and apoptosis. AAA lesion Ki67<sup>+</sup> proliferating cells were reduced in *Ctsl*<sup>-/-</sup> mice at 7 days and 14 days postperfusion (A; representative images are shown to the right), but cathepsin L deficiency did not affect AAA lesion cell apoptosis (B) or lesion medial smooth-muscle cell loss (C). The number of mice per group is indicated in each bar. Measurements in panels A and B are from the entire lesion including adventitia and media. All data are mean±SE.  $P < 0.05$  was considered statistically significant; Mann-Whitney U test.

**Figure 5.**

Medial elastin fragmentation and protease expression. A, Medial elastin fragmentation was reduced in abdominal aortic aneurysms (AAA) lesions from *Ctsl*<sup>-/-</sup> mice. The number of mice per group is indicated in each bar. B, RT-PCR demonstrated altered cathepsin and matrix metalloproteinases (MMP) transcript levels in aortic smooth-muscle cell from *Ctsl*<sup>-/-</sup> mice compared with those of *Ctsl*<sup>+/+</sup> mice. All data are mean±SE.  $P < 0.05$  was considered statistically significant; Mann-Whitney U test. C, In situ fluorescent zymogram assay showed high elastinolytic cysteinyl cathepsin activities in the adventitia of *Ctsl*<sup>+/+</sup> mouse AAA lesions (top left) that can be largely inhibited by 20 μmol/L of E64d (top right). Such activities were reduced in AAA lesion adventitia from *Ctsl*<sup>-/-</sup> mice (bottom left), and E64d further reduced cathepsin activities (bottom right). The percentages of adventitia fluorescence yielded from fluorogenic elastin degradation represented cysteinyl cathepsin activities. The white dotted lines separate medial elastin autofluorescence from the adventitia. D, Immunohistological analysis showed reduced medial expression of cathepsin S (top panels) and cathepsin K (bottom panels) in AAA lesions from *Ctsl*<sup>-/-</sup> mice, compared with those from *Ctsl*<sup>+/+</sup> mice.





**Figure 6.**

Periaortic  $\text{CaCl}_2$  injury-induced abdominal aortic aneurysms (AAA). A, Aortic diameter increase in percentage at 4 weeks after injury. B, Medial elastin fragmentation grades, as determined according to previously published keys (Ref. 19). Representative figures are shown to the right. Data are mean $\pm$ SE.  $P < 0.05$  is considered statistically significant, Mann-Whitney U test. The number of mice for each group is indicated in the bar.