Laminar Shear Stress Inhibits Cathepsin L Activity in Endothelial Cells

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Objective—The cysteine proteases, cathepsins, have been implicated in vascular remodeling and atherosclerosis, processes known to be regulated by shear stress. It is not known, however, whether shear regulates cathepsins. We examined the hypothesis that shear stress regulates cathepsin activity in endothelial cells.

Methods and Results—Mouse aortic endothelial cells (MAECs) exposed to atheroprotective, unidirectional laminar shear (LS) degraded significantly less BODIPY-labeled elastin and gelatin in comparison to static and proatherogenic oscillatory shear (OS). The cathepsin inhibitor E64 also reduced this activity. Gelatin zymography showed that cathepsin activity of MAECs was blunted by LS exposure and by a cathepsin L inhibitor but not by cathepsin B and S inhibitors, whereas a cathepsin K inhibitor had a minor effect. Cathepsin L siRNA knocked down cathepsin L expression, gelatinase, and elastase activity in OS and static MAECs. A partial reduction of cathepsin B protein raised the possibility that the siRNA effect on the matrix protease activity could have been attributable to cathepsin L or B. Cathepsin B activity study using the synthetic peptide showed it was not regulated by shear.

Conclusions—These results suggest that cathepsin L is a shear-sensitive matrix protease and that it may play an important role in flow-mediated vascular remodeling and atherogenic responses. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: shear stress ■ cathepsin ■ elastase ■ gelatinase ■ atherosclerosis

Vascular endothelial cells are constantly exposed to fluid shear stress, the frictional force generated by blood flow over the vascular endothelium. The importance of shear stress in vascular biology and pathophysiology has been highlighted by the focal development patterns of atherosclerosis in hemodynamically defined regions. For example, the regions of branched and curved arteries exposed to disturbed flow conditions including oscillatory and low mean shear stresses (OS) correspond to atheropone areas. In contrast, straight arteries exposed to pulsatile high levels of laminar shear stress (LS) are relatively well protected from atherosclerotic plaque development.1

Changes in blood flow have been shown to be a critical factor inducing arterial remodeling.1-3 Increases in arterial wall shear stress prevent vascular remodeling leading to thickening of the vascular wall and inflammation,2 whereas decreases in arterial wall shear stress promote arterial remodeling and inflammation.2,3 Additionally, low wall shear stress leads to degradation of the internal elastic lamina (IEL).5 Despite these findings, the underlying mechanisms by which shear regulates proteases degrading vessel wall matrix and IEL are not well described. Although there is a report demonstrating that shear regulates matrix metalloproteases (MMPs) in endothelial cells,8 it is not clear whether other proteases are also regulated by shear.

Cathepsins are the papain family of cysteine proteases which degrade elastin in addition to collagen.9 Unlike MMPs, the role for cathepsins in blood vessel remodeling and cardiovascular disease has been understudied until recently. Cathepsins K, L, and S, potent elastinolytic proteases, have been identified in atherosclerotic plaques10,11 and in neointima after balloon angioplasty.12 Furthermore, cathepsins B, L, and S also have been shown to be upregulated at the transcriptional level in the arteries of apolipoprotein E-null mice fed an atherogenic diet.13 In addition, cathepsin activity is increased in abdominal aortic aneurysms (AAA).10,14 Cathepsin L is classified as one of the potent mammalian collagenases and elastases15-18 and is capable of cleaving mature insoluble elastin.17 However, cathepsin L expression and its role in endothelial cells and atherosclerosis development are not well known.

Here, we hypothesized that shear stress regulates cathepsin activities in endothelial cells. We examined the effects of OS and LS on matrix proteolytic activities and cathepsin activity in endothelial cells. Our results show that LS reduces matrix protease activity in a cathepsin L-dependent manner.

Materials and Methods

Mouse Aortic Endothelial Cell Culture and Shear Stress Studies

Mouse aortic endothelial cells (MAEC) obtained from the thoracic aortas of C57/BL6 mice were isolated, cultured in growth medium...
(Dubbeco modified Eagle’s medium [DMEM] containing 20% fetal bovine serum [FBS], 100 µg/mL endothelial cell growth supplement [ECGS, Sigma], and 2.5 U/mL heparin) as described, and used between passages 7 to 10. Confluent endothelial monolayers grown in 100-mm tissue culture dishes were exposed to an arterial level of unidirectional LS (15 dyn/cm²) or OS with directional changes of flow at 1 Hz cycle (±5 dyn/cm²) for 1 day by rotating a Teflon cone (0.5° cone angle) as described previously. One hour before shear, the monolayers were washed and changed to 10 mL of fresh shear medium (the growth medium without serum).

**Elastase and Gelatinase Assay**

Five µg/mL of BODIPY fluorescein-conjugated DQ elastin or gelatin (Molecular Probes) in 5 mL of fresh serum-free DMEM was incubated with MAEC after exposure to OS, LS, or St for one day in the presence or absence of the cathepsin inhibitor E-64 (Sigma). After an additional 24 hours, aliquots (200 µL) of conditioned media were assayed with a fluorescence plate reader, in triplicate, with background fluorescence subtracted from the no-cell negative control at 485 nm excitation and 525 nm emission.

**Cathepsin Zymography**

Conditioned media were concentrated 20- to 30-fold with a spin concentrator (5-kDa cutoff; Vivascience), and protein concentration was determined with a fluorescence plate reader (excitation at 360 nm and emission at 460 nm). The conditioned media were concentrated as above. Equal amounts of total protein were resolved by SDS-PAGE, and the blots were probed with antibodies to cathepsins L (1:500; R&D), K (1:200; Calbiochem), B (1:250; Calbiochem), and S (1:1000; Santa Cruz), or β-actin (1:1000; Santa Cruz), and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method.

**Cysteine Protease Active Site Labeling**

Conditioned media were normalized by volume and an equal aliquot (20 µL) was incubated with a modified Lowry assay. Equal amounts of protein were resolved by 12.5% SDS-polyacrylamide gels containing 0.2% gelatin at 4°C. Proteins were renatured in 50 mM Tris buffer, pH 7.4 with 20% glycerol, and incubated overnight in assay buffer containing 0.1 mol/L sodium acetate buffer, pH 5.5, 1 mol/L EDTA, and 2 mol/L dithiothreitol in the presence or absence of cathepsin inhibitors: B (1 µmol/L CA074), L (1 µmol/L Z-FY buc)-DMK), K (1 µmol/L 1,3-Bis(CBZ-Leu-NH)-2-propanone, Calbiochem), or S (1 nM µ-Leu-Hph-VS-Phe). Gels were then rinsed with deionized water, stained with Coomassie Blue and destained, and analyzed by densitometry.

**Cathepsin Activity Assay**

Cells were lysed in 40 mmol/L sodium acetate buffer, pH 5.5, 0.1% Triton-X 100, and conditioned media were collected and concentrated. Aliquots were added to a reaction mixture containing 100 mmol/L L-lysine, pH 5.5, 2.5 mmol/L EDTA, 2 mmol/L dithiothreitol, and 0.1% Brij 35. Benzyloxycarbonyl-Arg-Arg-7-amino-4-methylcoumarin (Z-RR-AMC) (Biomol) was used as the substrate and added to obtain a final concentration of 5 µmol/L after the cathepsins were activated for 2 minutes at 37°C. The reaction mixture was incubated at 37°C for 10 minutes, and AMC fluorescence intensity was determined with a fluorescence plate reader (excitation at 360 nm and emission at 460 nm).

**Western Blots**

After shear, cells were lysed with RIPA buffer and conditioned media concentrated as above. Equal amounts of total protein were resolved by SDS-PAGE, and the blots were probed with antibodies to cathepsins L (1:500; R&D), K (1:200; Calbiochem), B (1:250; Calbiochem), and S (1:1000; Santa Cruz), or β-actin (1:1000; Santa Cruz), and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method.

**Transfection of siRNA**

Sub-confluent (75% to 80%) MAECs were transfected with annealed siRNA duplex [sense: 5′-UCAGUGGAGACCAAGUCAAtt, antisense: 5′-UGAGCUUGGAUCC UCAAUUGAt] or nonsilencing duplex [sense: 5′-UUCUGGAACGUCGCAGUt, antisense: 5′-ACGUGACACGUGGAGAAt] (Qiagen) using Oligofectamine (Invitrogen) in serum-free medium. After 6 hours, the medium was supplemented with serum (final 10% concentration) and cultured an additional 18 hours before exposing the cells to OS, LS, or no flow conditions.

**Statistical Analysis**

Student unpaired t-test was used to establish significance between groups. *P<0.05 was considered statistically significant.

**Results**

**LS Decreases Cell-Associated Extracellular Matrix Proteolytic Activity in Endothelial Cells**

To determine whether shear stress affected protease activities toward components of the extracellular matrix, we used BODIPY-gelatin and -elastin, soluble, fluorescently-labeled gelatin and elastin, as matrix substrates to live endothelial cells that had been exposed to 24 hours of OS, LS, or no flow (static) conditions. LS exposure significantly lowered both gelatinase and elastase activities in MAECs in comparison to those of static and OS-exposure (Figure 1A and 1B). In contrast, OS exposure had mixed effects on the matrix protease activities: OS increased the gelatinase activity by 28% above that of the static control, but did not affect the elastase activity (Figure 1A and 1B). Next we examined how much of the total matrix protease activity was contributed by cathepsins using the cathepsin inhibitor E64. Treatment with E64 inhibited the gelatinase and elastase activities by 30% to 50% in the static and OS-exposed cells. On the other hand, E64 did not have significant inhibitory effects on the gelatinase and elastase activities of LS-exposed cells (Figure 1A and 1B). These results raise a possibility that LS and E-64 target the same proteases, cathepsins that may be novel members of the mechanosensitive matrix proteases.
Endothelial Cells Exposed to LS Have Lower Cathepsin Activity Than That of OS

Next, we determined whether the mechanosensitive cathepsin activities are secreted into the conditioned media or remain associated with the cells. For this study, conditioned media were collected from MAECs that were exposed to OS, LS, or static conditions as in Figure 1, conditioned media (A) and cell lysates (B) were collected, and equal protein amounts were assayed by gelatin zymography optimized for cathepsins. Representative zymograms show gelatinolytic activities at 23/25 kDa bands and densitometric quantification is shown in the bar graphs (mean±SEM, n=7 to 9, *P<0.05). C. Equal volume of the conditioned media were labeled with the biotinylated active probe DCG-04 (5 μmol/L), resolved by SDS-PAGE, and the blot developed with a streptavidin-HRP method. Purified and denatured (boiled) cathepsin L were used as positive and negative controls, respectively. An additional control, the biotin blots were reprobed with a cathepsin L antibody. The biotin blot was quantified by densitometry as shown by the bar graph (n=3, P<0.001).

Figure 2. MAECs exposed to LS have lower cathepsin activity than that of OS. After 1 day of exposure of MAECs to LS, OS, or static conditions as in Figure 1, conditioned media (A) and cell lysates (B) were collected, and equal protein amounts were assayed by gelatin zymography optimized for cathepsins. Representative zymograms show gelatinolytic activities at 23/25 kDa bands and densitometric quantification is shown in the bar graphs (mean±SEM, n=7 to 9, *P<0.05). C. Equal volume of the conditioned media were labeled with the biotinylated active probe DCG-04 (5 μmol/L), resolved by SDS-PAGE, and the blot developed with a streptavidin-HRP method. Purified and denatured (boiled) cathepsin L were used as positive and negative controls, respectively. An additional control, the biotin blots were reprobed with a cathepsin L antibody. The biotin blot was quantified by densitometry as shown by the bar graph (n=3, P<0.001).

Figure 3. LS exposure inhibits cathepsin L activity in MAECs. A, After 1 day of exposure of MAEC to OS, LS, or static conditions, conditioned media were collected and analyzed by the cathepsin gelatin zymography in the absence or presence of the inhibitors of cathepsin B (1 μmol/L CA074), L (1 μmol/L Z-FY(t-Bu)-DMK), K (1 μmol/L 1,3-Bis(CBZ-Leu-NH)-2-propanone), or S (1 nM μ-Leu-Hph-VS-Ph). Shown zymograms are representative of at least 3 separate experiments. Equimolar active amounts of purified cathepsins L, B, K, and S were used in cathepsin gelatin zymography (B) and DCG-04 active cathepsin labeling (C). Second, the active protease activity associated with the 23/25 kDa proteins found in the conditioned media was further confirmed by an independent assay using DCG-04, which binds to active cathepsins. As shown in Figure 2C, the conditioned media obtained from OS-exposed MAECs contained significantly higher amounts of active cathepsins with ~25 kDa size in comparison to that of LS exposure. As positive and negative controls, the purified active cathepsin L and the inactive (boiled) enzyme were used. As expected, only the active form of cathepsin L but not the boiled enzyme bound to the DCG-O4 label (Figure 2C). Western blot of the same membrane showed that the amount of cathepsin L in the nonconcentrated conditioned media of MAECs was not sufficient to be detected, although the DCG-O4 label clearly identified the enzymes. These results suggest that shear-sensitive cathepsin activity is secreted into the media as detected by the more sensitive assays (zymography and the DCG-O4 labeling study), although the Western blot study was not sensitive enough to show the enzyme identity.

Cathepsin L Activity Is Regulated by Shear Stress in Endothelial Cells

To further determine which cathepsin(s) was responsible for the shear-dependent matrix protease activity, we used 4 cathepsin inhibitors during the cathepsin gelatin zymography assay. Again, the conditioned media of MAECs exposed to LS contained significantly reduced gelatinase activity in comparison to that of OS and static cells (Figure 3A). The inhibitors of cathepsin B (CA074) and S (μ-Leu-Hph-VS-Ph) had no effect on the gelatinolytic activity, whereas cathepsin K inhibitor [1,3-Bis(CBZ-Leu-NH)-2-propanone] showed a minor inhibitory effect (Figure 3A). The cathepsin L inhibitor [Z-FY(t-Bu)-DMK], however, completely blocked the gelatinase activity of the conditioned media from both OS and static-exposed cells.

In this gelatin zymography study, there was a possibility that the reason we observed only cathepsin L–like activity...
may have been because of a bias in assay conditions. For example, the zymography requires renaturation of the cathepsins after nonreducing SDS-PAGE. If for any reason cathepsins do not properly renature, we would not be able to detect their activities. To address this question, we loaded a gelatin gel with equivalent amounts of purified cathepsins L, B, K, and S based on their cathepsin activity assays using the peptide substrate Z-FR-AMC and E-64 titration curve.24 Of the 4 enzymes, only cathepsin L, but not B, K, and S, was capable of degrading the gelatin (Figure 3B). Active site labeling of the cathepsins showed that all 4 of the cathepsins were present in their active state (Figure 3C). These results show that the cathepsin zymography condition used in this study is sufficient for cathepsin L activity while the other cathepsins may not be active, possibly because of their failure to be renatured during the zymography assay. Based on these results, we cannot rule out whether cathepsins B, K, and S are mechanosensitive matrix enzymes or not. Nevertheless, the pharmacological results suggest that cathepsin L activity in the conditioned media is a mechanosensitive matrix protease.

**Cathepsin L siRNA Knocks Down Cathepsin L Protein and Significantly Reduces Endothelial Gelatinase and Elastase Activity**

To definitively address whether cathepsin L is a shear-sensitive matrix protease, we used a siRNA approach. Treatment of MAECs with cathepsin L siRNA knocked down cathepsin L protein expression more than 80% below that of nonsilencing controls in static, OS, and LS-exposed cells as shown in Western blots of the conditioned media. To examine the specificity of the siRNA against cathepsin L, we immunoblotted the cell lysates with antibodies for cathepsins B, K, and S. Cathepsin L siRNA did not cause nonspecific knockdown of cathepsin K and S (Figure 4A). However, it reduced cathepsin B protein expression by ~50%; cathepsin B exists as a 31-kDa single chain that is then processed into a 25/26-kDa double chain as reported by Linebaugh et al.25

Under identical conditions, cathepsin L siRNA treatment of MAECs blocked the cathepsin L activity stimulated by OS in the conditioned media (Figure 4B). This result provides strong evidence that the shear-sensitive gelatinolytic activity detected by the zymography is indeed cathepsin L. Additionally, cathepsin L siRNA treatment of the cells significantly inhibited the static and OS-induced gelatinase and elastase activities as determined by the degradation of BODIPY-gelatin and -elastin. Cathepsin L siRNA significantly inhibited both gelatinase and elastase activities of static and OS groups, although it tended to show a greater inhibitory effect on the elastase activity than the gelatinase (Figure 4C versus 4D). Cathepsin siRNA significantly reduced the gelatinase activity of static and OS groups by 25 and 30% of their nonsilencing static and OS controls, respectively (Figure 4C). In a similar trend, the siRNA significantly inhibited the elastase activity by 50 and 40% of the nonsilencing control static and OS groups, respectively (Figure 4D). In contrast, the cathepsin L siRNA had no significant effect on the gelatinase or the elastase activity in cells exposed to LS. These results are consistent with the E-64 results (Figure 1), suggesting that cathepsin L is an important shear-dependent matrix protease.

**Shear Stress Does Not Affect Cathepsin B Activity in Endothelial Cells**

With the above cathepsin L siRNA results, there remained a question whether the reduction in the gelatinase and elastase activities by cathepsin L siRNA treatment was caused by an unexpected partial knockdown of cathepsin B. Therefore, we decided to examine whether cathepsin B is a shear-sensitive protease. For this purpose, we used the cathepsin B specific peptide substrate Z-RR-AMC to assess cathepsin B activity. The results showed that neither LS nor OS significantly changed cathepsin B activities from that of the static conditions in either the cell lysates (Figure 5A) or conditioned media (Figure 5B) obtained from MAECs. Next, we examined whether the cell-permeable cathepsin B inhibitor (CA074Me) would reduce the cell-associated elastase activity. For this study, we treated MAECs with CA074Me at 0.1 μmol/L, a concentration that inhibits cathepsin B activity by ~70% (Figure 5C) without significantly affecting purified cathepsin L activity (data not shown). At this concentration, the cathepsin B inhibitor had no effect on the cell-associated elastase activities of the cells exposed to the static, OS, and LS conditions (Figure 5D). These results not only show that cathepsin B activity is not regulated by shear stress, it also

\[ \text{Figure 4. Cathepsin L siRNA knocks down cathepsin L protein and reduces endothelial cell-associated elastase activity. Subconfluent MAECs were transfected with cathepsin L specific siRNA (100 nM) or nonsilencing RNA (non siRNA) 24 hours before shear exposure. Transfected cells were then exposed to OS, LS, or static conditions for 1 day. A, Cathepsin L protein knockdown by cathepsin L siRNA was confirmed by Western blot using conditioned media and the cathepsin L antibody with Coomassie staining of the gel as a loading control. Cell lysates were collected and probed with antibodies to cathepsins B, K, and S, and an actin antibody as an internal control. B, Conditioned media obtained from A were examined by gelatin zymography as in Figure 2. The arrows indicate cathepsin L bands. The cell-associated gelatinase (C) and elastase (D) activities were determined using BODIPY-gelatin and -elastin as described in Figure 1. Shown are mean±SEM, (*P<0.05, n=4). ns: P>0.05.} \]
The role for cathepsins and their inhibitor cystatin C in elastic lamina degradation, vascular remodeling, and atherosclerosis has been demonstrated in animal models and humans. Mice deficient in cathepsin S in LDL receptor–null mice show decreased IEL fragmentation and reduction in atherosclerosis. Increased cathepsin L expression and decreased cystatin C levels in human AAA but almost no detectable cystatin C deficiency in apoE-null mice resulted in increased elastic lamina fragmentation and collagen content, which could have contributed to the dilation of thoracic and abdominal aortas. It remains controversial whether cystatin C deficiency affects atherosclerosis. Increased cathepsin L expression and decreased cystatin C have been found in human atherosclerotic plaques and aortic aneurysms.

The differential effects of laminar and oscillatory shear stresses on cathepsin L activity reported in this study may be a critical mechanism by which AAA occurs in regions of disturbed flow. Several human and animal studies have demonstrated that atherosclerotic lesions and aneurysms of the abdominal aorta occur in the regions where they are exposed to unstable flow conditions including flow reversal, low mean wall shear stress, and high oscillatory shear indices. In contrast, relatively high levels of laminar shear stress were shown to reduce AAA progression in rat experimental models, and a recent report found increased cathepsin L levels in human AAA but almost no detectable cathepsin L in normal arteries. Together, these previous findings and our current study raise an interesting possibility that differential regulations of cathepsin L by undisturbed and disturbed flow conditions may play a critical role in the protection or initiation and progression of AAA.

Shear stress potently regulates vascular remodeling, including the sizes of lumen and IEL fenestrae, and flow-dependent arterial remodeling is endothelium-
dependent. Although the mechanisms controlling cathepsin activities in smooth muscle cells have been reported, the role of cathepsins in endothelial cells have been rather limited. Shi et al showed that cathepsin S deficiency led to abnormal angiogenic responses attributable to abnormal extracellular matrix degradation. Also, cathepsin L has been shown to play an important role in endothelial progenitor cell–mediated neovascularization. Although shear stress has been shown to increase cathepsin B activity in neutrophils, the current study is the first report showing that cathepsins are regulated by shear stress in endothelial cells. The reason that cathepsin B is shear-sensitive in neutrophils, but not in endothelial cells as we showed here, may be attributable to unique cell-specific differences.

Shear stress has been shown to regulate another family of matrix proteases, MMPs both in cultured endothelial cells and in animal models. OS, but not LS, significantly stimulates matrix proteases, MMPs both in cultured endothelial cells and neutrophils, but not in endothelial cells as we showed here, that cathepsins are regulated by shear stress in endothelial cells. Shear stress regulates structure and function of endothelial cells and plays an important role in atherosclerosis development. The atheroprotective LS may protect the integrity of elastic lamina and extracellular matrix by inhibiting cathepsins such as L, whereas the proatherogenic OS have opposite effects. In summary, we showed that cathepsin L is a mechanosensitive matrix protease with a potential importance in vascular remodeling and atherosclerosis.

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Disclosure(s)
None.

References


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