Augmented Expression and Activity of Extracellular Matrix-Degrading Enzymes in Regions of Low Endothelial Shear Stress Colocalize With Coronary Atheromata With Thin Fibrous Caps in Pigs

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Background—The molecular mechanisms that determine the localized formation of thin-capped atheromata in the coronary arteries remain unknown. This study tested the hypothesis that low endothelial shear stress augments the expression of matrix-degrading proteases and thereby promotes the formation of thin-capped atheromata.

Methods and Results—Intravascular ultrasound–based, geometrically correct 3-dimensional reconstruction of the coronary arteries of 12 swine was performed in vivo 23 weeks after initiation of diabetes mellitus and a hyperlipidemic diet. Local endothelial shear stress was calculated in plaque-free subsegments of interest (n=142) with computational fluid dynamics. At week 30, the coronary arteries (n=31) were harvested and the same subsegments were identified. The messenger RNA and protein expression and elastolytic activity of selected elastases and their endogenous inhibitors were assessed. Subsegments with low preceding endothelial shear stress at week 23 showed reduced endothelial coverage, enhanced lipid accumulation, and intense infiltration of activated inflammatory cells at week 30. These lesions showed increased expression of messenger RNAs encoding matrix metalloproteinase-2, -9, and -12, and cathepsins K and S relative to their endogenous inhibitors and increased elastolytic activity. Expression of these enzymes correlated positively with the severity of internal elastic lamina fragmentation. Thin-capped atheromata developed in regions with lower preceding endothelial shear stress and had reduced endothelial coverage, intense lipid and inflammatory cell accumulation, enhanced messenger RNA expression and elastolytic activity of MMPs and cathepsins, and severe internal elastic lamina fragmentation.

Conclusions—Low endothelial shear stress induces endothelial discontinuity and accumulation of activated inflammatory cells, thereby augmenting the expression and activity of elastases in the intima and shifting the balance with their inhibitors toward matrix breakdown. Our results provide new insight into the mechanisms of regional formation of plaques with thin fibrous caps. (Circulation. 2011;123:621-630.)

Key Words: atherosclerosis • shear stress • endothelium • inflammation • proteases

Despite the systemic nature of risk factors for atherosclerosis, lesion distribution is geometrically focal and heterogeneous. Multiple atherosclerotic lesions at different stages of progression commonly coexist in an individual artery. Some atherosclerotic lesions with thin fibrous caps appear particularly prone to acute disruption and precipitation of an acute coronary syndrome. These lesions often do not limit blood flow and therefore are not detected or specifically treated before they rupture.

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Histopathological studies in animal models of atherosclerosis have demonstrated that such atherosclerotic lesions with high-risk characteristics preferentially develop in regions of
particularly low endothelial shear stress (ESS). However, the molecular mechanisms and the triggering pathophysiological determinants responsible for the local formation of plaques with thin fibrous caps remain unknown. Prior in vitro and ex vivo experiments have indicated that low ESS can augment the expression and activity of matrix-degrading proteases such as matrix metalloproteinase (MMP)-2 and MMP-9 and cathepsins K and L. These enzymes participate in the pathobiology of atherosclerosis in that they promote the degradation of the extracellular matrix macromolecules in the arterial wall and fibrous cap, facilitating the transmigration of inflammatory cells and vascular smooth muscle cells into the plaque. However, most of these studies used cultured cells, which lack the complex environment of the atherosclerotic vascular wall in vivo. The in vivo role of low ESS in the expression and activity of matrix-degrading proteases and their subsequent involvement in the development of atheromatous lesions with thin fibrous cap remains unknown.

This study tested the hypothesis in vivo that low ESS augments messenger RNA (mRNA) expression and activity of elastolytic proteases in coronary atherosclerotic plaques relative to their endogenous inhibitors, thereby shifting the plaque to a more elastolytically active state and facilitating the progression and differentiation of that plaque to an atheroma with thin fibrous cap. We studied pigs with advanced atheromatous lesions similar to those observed in humans.

**Methods**

**Porcine Model of Atherosclerosis**

A detailed description of the methods is presented in the online-only Data Supplement. Briefly, 12 male Yorkshire swine were rendered diabetic through streptozotocin injection and fed a high-fat diet. Twenty-three weeks (baseline) after the induction of diabetes mellitus and initiation of the high-fat diet, the pigs underwent in vivo vascular profiling of all the major epicardial coronary arteries to assess the local ESS along the surface of the reconstructed lumen, as previously described. At week 30 (follow-up), the animals underwent repeat vascular profiling and were euthanized, and the coronary arteries were harvested and assessed histopathologically.

**In Vivo Vascular Profiling**

ESS was calculated on the lumen surface of the reconstructed coronary arteries at baseline and correlated with the histopathology, mRNA and protein expression, and elastolytic activity of matrix-degrading enzymes at follow-up. We selected 142 arterial subsegments of interest 3 mm in length on the basis of different levels of baseline ESS magnitude. The selected subsegments exhibited varied 3-dimensional geometry. We focused on subsegments free of apparent atherosclerosis by intravascular ultrasound at baseline (maximum intima-media thickness ≤0.5 mm by intravascular ultrasound) because this allowed us to study the role of ESS in the initiation and progression of coronary atherosclerosis through multiple natural history stages, culminating in severe plaques with features found in ruptured plaques in humans.

Baseline ESS was measured with computational fluid dynamics as previously described. Local ESS was averaged in each 3-mm-long subsegment and classified as either low ESS (1.0 Pa; n = 79; mean, 0.67 ± 0.24 Pa) or higher ESS (≥1.0 Pa; n = 63; mean, 1.59 ± 0.07 Pa). The arterial remodeling response to plaque formation was estimated at follow-up by intravascular ultrasound in each subsegment and classified as compensatory expansive, expansive, or constrictive.

**Histopathological, mRNA, and In Situ Zymography Analyses**

The subsegments were located in the harvested coronary arteries by identifying several major and readily visible side branches on the ESS and wall thickness maps, as well as on the harvested arteries. The middle portion of the subsegments was cryosectioned at 7-μm thickness, Verhoeff elastin and Oil Red O staining, as well as CD31 and CD45 immunostaining, were performed in each cryosection for the assessment of intima-to-media ratio (IM), lipid accumulation, luminal endothelial cell coverage, and inflammatory cell infiltration, respectively. We performed immunostaining for the class II histocompatibility complex (MHC-II) molecules in serial sections with CD45 immunostaining to assess the plaque content of activated inflammatory cells. For quantification of all histological assays, we measured the percent of the intima with positive staining.

mRNA was harvested from the intima and media of the subsegments. mRNA encoding selected matrix-degrading elastolytic proteases (ie, MMP-2, MMP-9, MMP-12, and cathepsins K, L, and S) and their inhibitors (ie, tissue inhibitor of MMP [TIMP]-1, TIMP-2, and cystatin C) were measured by real-time reverse-transcriptase polymerase chain reaction (Table I in the online-only Data Supplement). The protein expression of MMP-2 and cathepsin-S was assessed by immunohistochemistry and immunofluorescent staining in representative subsegments. The MMP- and cathepsin-mediated elastolytic activity was assessed and quantified by in situ zymography (ISZ).

Lesions at follow-up were classified histopathologically into 3 categories: minimal lesions, defined as lesions with IM <0.15; intermediate lesions, defined as lesions with IM ≥0.15 without evidence of fibrous cap; and thin-capped atheroma, defined as lesions with IM ≥0.15 with a thin (<65 μm) fibrous cap overlying a lipid core. The integrity of the internal elastic lamina (IEL) was assessed in Verhoeff elastin-stained sections and classified into 4 grades. The integrity of the IEL was correlated with the ESS at baseline and with the subsequent elastase expression and activity at follow-up.

**Statistical Analyses**

All analyses were performed with SPSS 17.0 (SPSS Inc, Chicago, IL) and Stata 10.0 (StataCorp LP, College Station, TX). Continuous variables are summarized as mean ± SEM and categorical variables as actual numbers and percentages. To correct for systematic error introduced by the clustering of arterial subsegments within animals, several statistical methods were used. First, to investigate the association of continuous dependent variables (eg, CD31-positive endothelial cells, expression of matrix-degrading enzymes, content of activated macrophages) with categorical independent variables (eg, baseline ESS), mixed-effects ANOVA with the animal as random effect was used. Although ESS is a continuous variable, these analyses were performed by dichotomizing ESS as a categorical variable. Second, for analyses with continuous dependent (eg, expression of matrix enzymes) and continuous independent variables (eg, histopathological characteristic, cholesterol levels), linear regression was used. Finally, when the dependent variable was categorical (eg, lesion category, IEL grade), either ordinary or ordered logistic regression was used. In linear regression and logistic regression analyses, the SIs of the regression coefficient were adjusted for clustering of arterial subsegments within animals with the Huber White sandwich estimator. In all analyses, there were no missing values, and adjusted for clustering of arterial subsegments within animals with the Huber White sandwich estimator. In all analyses, there were no missing values, and adjustments were made for multiple comparisons of data with either the Scheffé or modified Bonferroni method. Findings were considered statistically significant at the 0.05 level.

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**

**Reduced CD31-Positive Endothelial Cells in Low-ESS Subsegments**

In subsegments with low baseline ESS, the percentage of luminal periphery with CD31-positive endothelial cells was...
significantly reduced compared with subsegments with higher baseline ESS (Figure 1A). Inflammation was negatively associated with the percent of luminal periphery with CD31-positive endothelial cells ($r=-0.65$, linear regression coefficient $-0.017$, SE $0.005$; $n=29$; $P=0.001$), supporting the crucial role of endothelial integrity in intimal inflammatory cell infiltration. Figure 1D through 1G shows CD31 immunofluorescence and CD45 immunostaining in representative lesions with low and higher baseline ESS, respectively.

Increased Expression and Activity of Elastolytic MMPs in Low-ESS Subsegments

Subsegments with low baseline ESS had significantly higher levels of mRNAs encoding MMP-2, -9, and -12, as well as their endogenous inhibitors TIMP-1 and -2, compared with subsegments with higher baseline ESS (Figure 2A). Despite the parallel increase in mRNA expression of both MMPs and their inhibitors in low-ESS subsegments, the ratio of MMP to TIMP was higher in low-ESS subsegments, indicating an increase in MMP over TIMP expression in low- versus higher-ESS subsegments.

To investigate the potential factors associated with the expression of the investigated proteases, we applied mixed-effects ANOVA using the mRNA expression of the enzymes as the dependent variable. The lowest baseline ESS and increased total cholesterol were both significant predictors of mRNA levels of the proteases (Table II in the online-only Data Supplement). These results indicate that both local ESS and the magnitude of hypercholesterolemia independently determine the expression of these enzymes.

To assess the protein expression of the investigated enzymes, we performed immunohistochemical staining for MMP-2 in selected subsegments of low versus higher base-

Increased Expression and Activity of Elastolytic Cathepsins in Low-ESS Subsegments

Similar to the observations of the elastolytic MMPs, subsegments with low baseline ESS had 1.5- to 2-fold greater levels of mRNA for cathepsins K and S, as well as their endogenous inhibitor cystatin C, than subsegments with higher ESS (Figure 3A). Furthermore, cathepsin-mediated elastolytic activity, assessed by ISZ optimized for cathepsins, was more...
pronounced in low-ESS subsegments and colocalized with CD45-positive inflammatory cells (Figure 3B through 3D). The zymographic activity, quantified as the percentage area of intimal fluorescence, was higher in subsegments of low versus higher ESS (32.4 ± 2.8% versus 11.6 ± 0.9%; P < 0.001; Figure 3D and 3G). Cathepsin-mediated zymographic activity was abolished by the addition of 10 mmol/L E64D, a sulfhydryl protease inhibitor, indicating the relative contribution of such enzymes to the zymographic signal (Figure 3E).

Double-immunofluorescent staining for CD45 and cathepsin S further confirmed the increased expression of cathepsin S, as well as the localization of cathepsin S protein, in CD45-positive inflammatory cells in subsegments of low baseline ESS (Figure 3H through 3J) versus subsegments of higher baseline ESS (Figure 3K).

**Enhanced Content of Activated Inflammatory Cells in Subsegments of Low ESS**

To evaluate the levels of inflammation in low- versus higher-ESS subsegments, we performed immunostaining for CD45-positive inflammatory cells. To assess the content of activated inflammatory cells, we also stained for MHC-II antigen as an indicator of activated leukocytes in response to proinflammatory cytokines such as interferon-γ. Although the percentage of intimal area infiltrated with inflammatory cells did not differ significantly (Figure 4A), lesions with low baseline ESS displayed a higher percentage of the intimal area positive for MCH-II staining (8.8 ± 1.2% versus 3.1 ± 0.5%; P < 0.001; Figure 4B and D through G). The relative proportion of inflammatory cells that were activated (MHC-II positive) was significantly elevated in plaques that originated from low ESS (43 ± 3.9% versus 20.3 ± 3.8%; P < 0.001; Figure 4C). These findings agree with the augmented expression and activity of matrix-degrading enzymes by activated inflammatory cells in low-ESS subsegments.

**Association of IEL Fragmentation With Increased Expression and Activity of Elastolytic Proteases**

Plaques with severe IEL fragmentation (ie, grades 2 and 3) developed in subsegments with lower baseline ESS and had higher levels of mRNAs encoding MMP-9 and cathepsin K and increased ratios of MMP to TIMP and cathepsin to cystatin C compared with lesions with no or minimal IEL fragmentation (ie, grades 0 and 1; Figure 5A through 5E).

**Figure 2.** Increased expression and activity of MMPs in low-ESS (n=79) vs higher-ESS (n=63) subsegments. A, Relative mRNA levels of MMP-2, MMP-9, MMP-12, TIMP-1, and TIMP-2 and the ratio of MMPs to TIMPs in low- vs higher-ESS subsegments. B, Representative immunostaining for CD45 in a low-ESS lesion. Higher magnification of (C) CD45 staining and (D) MMP-2 staining in the area selected by the black box in B. F, ISZ optimized for MMPs shows intense green fluorescence, indicating high elastolytic activity in serial sections with C and D. G, The MMP-inhibitor EDTA abolished elastolytic activity in the adjacent section. E, Absence of MMP-2 staining in a representative lesion of higher baseline ESS. H, Absence of MMP-mediated elastolytic activity in the same lesion as in E. Elastolytic activity–dependent fluorescence is shown in red in the insets in F through H. The percentage of intimal area with fluorescence intensity is indicated in each inset. I, Double immunofluorescence for (I) CD45 and (J) MMP-2 in a representative lesion of low ESS. K, Orange indicates colocalization of both antigens. L, Elastolytic activity (ISZ optimized for MMP) colocalizes with inflammatory cells infiltration (M, CD45 immunofluorescence applied in an adjacent section) in a lesion of low baseline ESS. N, Orange indicates colocalization. Asterisks indicate the lumen.
Consistent with these results, we found increased elastolytic activity of MMPs and cathepsins by ISZ in low-ESS plaques with severe IEL fragmentation versus higher-ESS plaques with intact IEL (Figure 5F and 5G). These results indicate the preponderance of active elastases relative to their inhibitors in lesions with low baseline ESS and enhanced subsequent IEL fragmentation.

Lesions with excessive expansive remodeling were associated with the lowest baseline ESS values and displayed larger lumen, external elastic membrane, and plaque volumes at follow-up (Figure I in the online-only Data Supplement). We also found a strong positive association of IEL fragmentation with excessive expansive remodeling ($P=0.005$; Table III in the online-only Data Supplement). Taken together, these results indicate that low baseline ESS is closely associated with the severity of IEL fragmentation and excessive expansive remodeling, likely through the increased expression and activity of elastolytic enzymes.

Figure 3. Increased expression and activity of cathepsins in low-ESS (n=79) vs higher-ESS (n=63) subsegments. A, Relative mRNA levels of cathepsins K and S and cystatin C in low- vs higher-ESS subsegments. B, Representative immunostaining for CD45 in a lesion of low baseline ESS. C, Higher magnification of the region selected by the black box in B. D, ISZ optimized for cathepsins shows intense green fluorescence, indicating high elastolytic activity, in the inflammatory cell-rich area. E, Addition of cathepsin inhibitor E64D eliminates the enzyme activity in the serial section. F, CD45 immunostaining and (G) ISZ in a representative lesion of higher baseline ESS. Elastolytic activity–dependent fluorescence is shown in red in the insets in D, E, and G. The percentage of intimal area with fluorescence intensity is indicated in each inset. Immunofluorescent staining for (H) CD45 and (I) cathepsin S in a representative lesion of low ESS shows the marked expression and colocalization of both antigens, indicated by the merge of H and I (J). K, Double-immunofluorescent staining for CD45 (green) and cathepsin S (red) in a subsegment of higher ESS. Asterisks denote the lumen.

Figure 4. Low-ESS subsegments contain more activated inflammatory cells compared with higher ESS subsegments. A, The percentage of CD45-positive intimal area did not differ between low-ESS (n=79) and higher-ESS (n=63) subsegments. However, low-ESS subsegments (n=32) had a higher percentage of MHC-II–positive intima area and a higher proportion of activated (MHC-II–positive) inflammatory cells compared with higher-ESS subsegments (n=24). Microphotographs represent (D) CD45 staining and (E) MHC-II staining in parallel sections of a low-ESS subsegment and a higher-ESS subsegment (F and G, respectively). Asterisks indicate the lumen.
Differential Expression of Matrix-Degrading Proteases in Lesions of Differing Morphologies

All lesions were histopathologically classified at follow-up (week 30) into 3 categories: minimal lesions (n=26, 18.3%), intermediate lesions (n=56, 39.4%), and atheromata with thin fibrous cap (n=60, 42.3%). As previously published, both thin-capped atheromata and intermediate lesions developed in subsegments with lower baseline ESS compared with minimal lesions (thin-capped atheromata, 0.91 ± 0.07 Pa; intermediate, 0.98 ± 0.06 Pa; minimal, 1.69 ± 0.12 Pa; P < 0.001). Of note, 72% of atheromata with thin caps developed at week 30 in subsegments with low baseline ESS (<1.0 Pa) at week 23, whereas 28% of atheromata with thin cap originated from arterial regions with particularly low baseline ESS (<0.6 Pa; Figure II in the online-only Data Supplement).

Thin-capped atheromata displayed reduced percent of lumen with CD31-positive endothelial cells (Figure 1B) and significantly increased mRNA levels of MMP-2 and MMP-9, as well as cathepsins K and S, compared with both minimal and intermediate lesions (Figure 6). Although thin-capped atheroma had increased levels of mRNAs encoding the protease inhibitors TIMP-1, TIMP-2, and cystatin C, the MMP-to-TIMP and cathepsin-to-cystatin mRNA ratios increased significantly, indicating a net increase in the elastolytic potential in atheromata with thin caps (Figure 6).

Hemodynamic, Histomorphological, and Molecular Determinants of the Development of Atheromata With Thin Fibrous Cap

We focused on a subgroup of 52 similarly sized lesions of intermediate histomorphology (IM, 0.3 to 0.9; Figure III in the online-only Data Supplement). These lesions were either small atheroma with thin fibrous cap (n=20), presumed precursors of advanced thin-capped atheromata of the type...
associated with fatally disrupted human plaques, or atheromata without evidence of fibrous cap (n=32). With this analysis, we assessed the relationship between prior hemodynamic environment (week 23) and the subsequent histopathological characteristics and protease profile (week 30) of comparably sized, intermediate-stage lesions to investigate the mechanisms that promote the evolution of each plaque type and the differential impact of different ESS magnitudes on plaque characteristic adjusting for the effect of ESS on plaque size.

These 2 lesion types had similar size (IM, 0.56±0.04 versus 0.53±0.03; P=0.44) and developed in pigs with similar hypercholesterolemia (628±14 versus 619±13 mg/dL; P=0.62) and hyperglycemia (216±19 versus 220±15 mg/dL; P=0.88). The local ESS preceding the development of small atheromata with thin fibrous cap tended to be lower than that in atheromata without fibrous cap (0.75±0.07 versus 1.00±0.09 Pa; P=0.08). There were marked differences between small atheromata with thin cap and atheromata without fibrous cap in terms of luminal CD31-positive endothelial cells (37±6% versus 72±3%; P<0.001; Figure 1C), relative lipid content (70±6% versus 53±4%; P=0.02), and relative inflammatory cells content (43±8% versus 27±4%; P=0.06). With regard to the protease profile, small atheromata with thin cap had twice as much mRNA levels of MMP-2 and MMP-9, increased cathepsins K and S mRNAs, and a higher ratio of cathepsin to cystatin C compared with atheromata without fibrous cap (the Table). Small atheromata with thin cap were also associated with greater IEL fragmentation compared with atheromata without fibrous caps (P=0.05).

**Discussion**

This study explored in vivo the effect of local baseline ESS on the expression and elastolytic activity of selected proteases implicated in atherosclerosis and their endogenous inhibitors to investigate the mechanisms governing the local development of thin-capped atheromata. We induced a range of advanced lesions in the coronary arteries of diabetic, hyperlipidemic swine, including atheromata with thin fibrous caps resembling those found in humans.3,10 In contrast to cell culture studies that examined protein expression under de-

**Table. mRNA Expression of Matrix-Degrading Enzymes and Their Inhibitors in Atheromata Without Fibrous Cap Versus Small Thin-Capped Atheromata**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Atheromata Without Fibrous Cap (n=32)</th>
<th>Small Thin-Capped Atheromata (n=20)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>0.028±0.005</td>
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<td>0.004</td>
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<tr>
<td>MMP-9</td>
<td>0.781±0.164</td>
<td>1.589±0.206</td>
<td>0.004</td>
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<tr>
<td>MMP-12</td>
<td>0.746±0.283</td>
<td>0.557±1.822</td>
<td>0.70</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.282±0.036</td>
<td>0.365±0.059</td>
<td>0.41</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.293±0.053</td>
<td>0.492±0.067</td>
<td>0.10</td>
</tr>
<tr>
<td>MMP/TIMP</td>
<td>1.429±0.202</td>
<td>2.604±0.343</td>
<td>0.07</td>
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<tr>
<td>Cathepsin K</td>
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<td>0.500±0.093</td>
<td>0.005</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>0.064±0.010</td>
<td>0.075±0.013</td>
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</tr>
<tr>
<td>Cathepsin S</td>
<td>0.363±0.067</td>
<td>0.682±0.093</td>
<td>0.023</td>
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<tr>
<td>Cystatin C</td>
<td>1.729±0.256</td>
<td>2.299±0.381</td>
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<tr>
<td>Total cathepsins/cystatin C</td>
<td>0.413±0.044</td>
<td>0.748±0.117</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Values represent mean±SEM.
fined shear, we directly evaluated the plaque morphology and protease profile in regions of the coronary vasculature with defined in vivo assessment of local ESS. For the first time, we show that lesions in regions exposed to low ESS had reduced endothelial cell coverage, augmented infiltration of activated inflammatory cells, and substantially increased expression and enzymatic activity of elastases relative to their inhibitors. These enzymes likely contribute to fragmentation of elastin fibers in the IEL and promote excessive expansive remodeling response of the vessel wall, ultimately promoting the formation of atheromata with thin fibrous cap.

Role of Low ESS in the Expression and Activity of MMPs and Cathepsins
A complex combination of collagen, elastin, and proteoglycans makes up the extracellular matrix of the arterial wall and atheroma and determines the integrity of individual plaque.

Prior cell culture studies have shown that low and oscillatory ESS augment the endothelial cell expression of MMPs that localize in atherosclerotic plaques.8,17 In vivo studies in the carotid arteries of mice demonstrated further that low ESS increases the expression and activation of MMPs in atherosclerotic plaques.5 These previous studies have not determined the relationship between low ESS and protease balance in the coronary arteries or in the intact animal. We now show that coronary arterial regions with low ESS develop lesions with greatly enhanced MMP-2, MMP-9, and MMP-12 expression and elastolytic activity. Although the exact role of MMP-9, particularly in plaque stability, rupture, and healing, is not yet conclusive,18 our present results are in accordance with existing clinical and experimental data that strongly support the role of MMP-9 in plaque destabilization and rupture.19

ESS regulates gene expression through several mechanisms, including activation of shear stress response elements in promoters and increased levels of the transcription factors KLF-2 and KLF-4.1 Subsequent production of proinflammatory cytokines and chemokines in the intima can in turn promote the accumulation and activation of inflammatory cells and augment protease release from their key cellular sources (ie, endothelial cells, macrophages, and vascular smooth muscle cells).8 Low ESS can also directly stimulate MMP gene expression in endothelial cells through activation of nuclear factor-κB17,20 TIMP gene expression also increases in atherosclerotic lesions exposed to low ESS, but our data indicate a net increase in proteolytic activity in these regions.

In addition to the MMPs, several other elastases, including cysteiny1 cathepsins, participate in extracellular matrix breakdown in atheromata.9 Cell culture studies have previously shown that low and oscillatory ESS augment the endothelial cell expression of cathepsins K and L.7 The present study provides novel in vivo evidence that coronary artery subsegments exposed to low ESS have enhanced mRNA and protein expression, as well as elastolytic activity, of cathepsins. Subsequent stimulation of low-ESS–mediated cytokine- and nuclear factor-κB–dependent pathways similar to those for MMPs appears to promote the expression of cathepsins, shifting the local arterial vascular wall toward a more elastolytic state.1

Pathobiological Mechanisms Responsible for the Evolution of Atheromata With Thin Fibrous Cap
We have previously shown that low ESS influences the evolution of coronary plaques toward lesions with characteristics of high risk for rupture4 and that the magnitude of low ESS associates with the development of late-stage thin-capped atheromata through enhancement of local inflammation.3 Little is known, however, about the molecular events that determine the evolution of an early or intermediate lesion to a late-stage thin-capped atheroma. We addressed this question of molecular pathobiological determinants by analyzing a subgroup of 52 similarly sized lesions at an intermediate stage of development. Our analyses demonstrated that early plaques with thin fibrous caps and more intense inflammation developed in arterial regions with lower ESS compared with those plaques with no fibrous cap. Furthermore, the inflamed thin-capped atheroma had markedly increased mRNA expression and elastolytic activity of matrix-degrading enzymes compared with atheroma without fibrous cap. These results establish that the local hemodynamic stimulus of low ESS closely associates with the expression of elastolytic proteases during the early and intermediate stages of atherosclerosis. At the molecular level, low ESS favors endothelial dysfunction and subsequent attenuation in the expression of the cassette of “atheroprotective” genes,1,21 influx and activation of inflammatory cells into the intima, production of extracellular matrix-degrading enzymes (including gelatinases and elastases), and intimal extracellular matrix dissolution. Products of intimal inflammatory cells can stimulate vascular smooth muscle cells to migrate from the media to the intima through IEL fenestrae22 and foster plaque growth and formation of the fibrous cap, which overlies the lipid core, yielding an early atheroma with fibrous cap. Through the IEL discontinuity, which also has been described in human coronary plaques,23 the inflammatory cells can also extend to the media where they elaborate matrix-degrading enzymes and promote expansive remodeling of the vascular wall. In such a locally expanded vascular region, the local ESS becomes even lower, as previously demonstrated,3,4 and initiates a self-perpetuating vicious cycle of low ESS, endothelial dysfunction, inflammation, matrix-degrading activity, and wall expansion.3,4 The intense matrix degradation may progressively drive the evolution of an early small atheroma to a high-risk thin-capped atheroma.

Although a majority (72%) of thin-capped atheromata developed in subsegments of low ESS, 28% of thin-capped atheromata originated from regions with higher baseline ESS. Furthermore, although the mRNA expression and activity of the matrix-degrading proteases were augmented in regions of low ESS, we found some heterogeneity in the expression of these enzymes even in regions of similarly low ESS. Taken together, our results suggest that low ESS clearly promotes high-risk plaque progression and augments the expression of the elastolytic proteases, but other local or systemic factors that were not analyzed in the present study such as the
severity of hypercholesterolemia, vascular remodeling, and wall structural characteristics may also be in play.24

High ESS may induce pathobiological responses within the plaque that exacerbate plaque fragility, as suggested by the association of high ESS with high strain, a possible marker of vulnerable plaque composition.25 Furthermore, human studies have shown that localized high shear stress may constitute a trigger for fibrous cap rupture.26 The nature and specific magnitude of ESS responsible for plaque rupture remain to be elucidated.

Study Limitations
The arterial subsegments we investigated were not randomly selected; instead, they were identified a priori on the basis of ESS distribution. We were careful to include subsegments across a spectrum of baseline ESS magnitudes but could still have introduced selection bias in our samples, especially because we were dealing with a restricted number of animals. Like all studies, use of a larger number of animals at multiple times would have been beneficial if feasible. The power of the study increased, however, by investigating multiple subsegments in each coronary artery (average, 5 subsegments per artery).

The fibrous cap thickness was measured in Oil Red O–stained sections. Although this is the standard method for assessing fibrous cap thickness in human arterial sections, it does not provide simultaneous staining of both the fibrous and lipid components of the plaque. In our analyses, we assumed that prominent staining for lipids with Oil Red O dye correlated with a loss of fibrous components in this region of the arterial section. A detailed description of the limitations of computational fluid dynamics and histopathological analyses is provided in the online-only Data Supplement.

Conclusions
Low ESS induces endothelial discontinuity and accumulation of activated inflammatory cells, thereby augmenting the expression and elastolytic activity of extracellular matrix-degrading proteases in the intima and shifting the balance with their inhibitors toward matrix breakdown. These mechanisms may contribute critically to extracellular matrix remodeling during the formation and evolution toward high-risk, rupture-prone coronary atherosclerotic plaque.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

This study explores the molecular mechanisms that determine the localized formation of thin-capped fibroatheromas in the coronary arteries of diabetic, hyperlipidemic swine. Local endothelial shear stress was calculated in vivo with a combination of intravascular ultrasound, coronary angiography, and computational fluid dynamics in plaque-free subsegments of interest at baseline (week 23). The messenger RNA and protein expression and the elastolytic activity of selected matrix-degrading proteases were assessed in these subsegments at follow-up (week 30), demonstrating that (1) subsegments with low preceding endothelial shear stress at week 23 had reduced endothelial cell coverage and enhanced lipid accumulation and inflammation at week 30; (2) these subsegments showed increased expression of messenger RNAs encoding matrix metalloproteinase-2, -9, and -12 and cathepsins K and S relative to their endogenous inhibitors and increased elastolytic activity; and (3) expression of these enzymes correlated positively with the severity of internal elastic lamina fragmentation. We further showed that thin-capped atheromata developed in regions with lower preceding endothelial shear stress and had reduced endothelial coverage, intense lipid and inflammatory cell accumulation, enhanced messenger RNA expression of matrix metalloproteinases and cathepsins, heightened elastolytic activity, and severe internal elastic lamina fragmentation. Our results provide new insight into the hemodynamic and molecular mechanisms of regional formation of plaques with thin fibrous cap and indicate that the in vivo understanding of local endothelial shear stress may allow identification of a high-risk plaque in its early stages of development. Early identification of a high-risk plaque may provide a rationale for selective local coronary interventions, supplemented by an intensive systemic pharmacological approach, to avert future acute coronary events.
Augmented Expression and Activity of Extracellular Matrix-Degrading Enzymes in Regions of Low Endothelial Shear Stress Colocalize With Coronary Atheromata With Thin Fibrous Caps in Pigs


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Study protocol

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Vascular profiling for ESS calculation at baseline

Intracoronary vascular profiling used methodology previously described and validated in-vivo.\textsuperscript{2-7} In brief, the 3D anatomy of the coronary artery was reconstructed from intravascular ultrasound (IVUS) images and biplane coronary angiography. Intravascular ultrasound (IVUS) (ClearView, Boston Scientific, Natick, MA) was performed with automated pullback at 0.5
mm/sec. The arterial lumen and external elastic lamina were segmented from digitized end-diastolic IVUS images. The physical 3D path of the IVUS transducer during pullback was reconstructed using the corresponding biplane angiographic projections, and the segmented IVUS images were located along this path and oriented appropriately. Lumen and external elastic membrane boundary points were connected by spline curves to rebuild the lumen and external elastic membrane geometry in 3D space, respectively. A structured grid was employed to represent the lumen volume. Coronary blood flow for the reconstructed arterial segment was calculated directly from the time required for the volume of blood contained within the segment to be displaced by radio-opaque material during a contrast injection. Blood was treated as a Newtonian fluid and its viscosity was estimated using the hematocrit and TC. Detailed intravascular flow characteristics were obtained by Computational Fluid Dynamics, solving the transport equations governing the conservation of mass and momentum (PHOENICS, Cham Ltd, London, UK). The governing equations of blood flow were determined assuming that the arterial wall is stiff, blood is incompressible, and coronary blood flow is steady. The reproducibility of our methodology of flow rate measurement has been previously reported. The mean flow rate in the 31 profiled arteries was 1.66±0.66 m/sec (range: 0.78-3.14 m/sec). The entrance velocity profile was not measured by our methodology, because all possible entrance velocity profiles converge to a single profile within about 3 mm of the origin. The inlet velocity was assumed to be uniform, developed flow was assumed to be established after an entrance length of 3mm, and flow was ignored for the first 3 mm. The distortions introduced by these assumptions were insignificant at the Reynolds numbers observed in this study. Endothelial shear stress at the lumen surface of the artery was calculated as the product of blood viscosity and the gradient of blood velocity at the wall.
Identification of arterial subsegments of interest at baseline and follow-up

To investigate the effect of ESS on mRNA and protein expression of matrix degrading enzymes, ESS was calculated on the lumen surface of the reconstructed coronary arteries at baseline (week 23) and correlated with the mRNA and protein expression at follow-up (week 30). Arterial subsegments of interest 3 mm in length were selected on the basis of different levels of baseline ESS magnitude. The subsegments exhibited varied 3D geometry from very straight to curved sections. To be considered in the analysis, each subsegment of interest was required to be free of apparent atherosclerotic plaque at baseline, defined by IVUS as maximum intima and media thickness $\leq 0.5$ mm, and to have a surface area of similar ESS $\geq 30\%$ of the total surface area of the subsegment.\textsuperscript{2,5} We focused on subsegments free of apparent atherosclerosis by IVUS at baseline (week 23) because this allowed us to study the role of ESS in the initiation and progression of coronary atherosclerosis through multiple natural history stages culminating in high-risk plaque. To reduce the impact of local flow disturbances on our results, subsegments free of side branches and located at least 1 mm away from adjacent side branches were selected.\textsuperscript{2}

The subsegments of interest were located in the preserved coronary arteries.\textsuperscript{2} To aid in locating each subsegment, several major and readily visible side branches were identified at baseline and follow-up on the ESS and wall thickness maps. The same side branches were also identified on the harvested arteries by utilizing magnifying lenses (2x). Using these branches as landmarks the exact location of each subsegment of interest was then identified on the preserved coronary arteries. The middle portion of the frozen subsegments of interest was cryosectioned at 7 $\mu$m thickness for histopathologic analysis, mRNA isolation and \textit{in-situ} zymography (ISZ). Endothelial shear stress which was averaged within a 3 mm long arterial subsegment was represented by a single histologic section derived from the middle portion of that subsegment.
However, selection of relatively short subsegments of interest, which exhibit homogeneity of hemodynamic and histopathologic characteristics along their length\(^8\), permitted accurate registration of the local ESS environment with the histopathologic findings.

**Histopathology**

The intima-to-media ratio (IM) was measured in Verhoeff’s elastin-stained cryosections by computerized planimetry. Lipid deposition and inflammatory cell infiltration were measured in oil red O- and CD45-stained cryosections. Immunostaining for CD45 used a monoclonal antibody (1:50, clone 2A5, BD Biosciences Inc., San Jose, CA) against pig CD45 leukocyte common antigen. Immunostaining for the class II histocompatibility complex (MHC-II) molecules (1:90, murine MHC class II, Pharmingen, San Diego, CA) provided an assessment of inflammatory cells activation in the atherosclerotic intima; parallel immunostaining for CD45 and MHC-II was used in n=56 available subsegments to evaluate the percentage of activated inflammatory cells.\(^9,10\) Oil red O-, CD45- and MHC-II positive intimal areas were assessed with a computer-assisted color-gated technique and are presented as percent of the intima.

Fibrous cap thickness was measured in Oil-red-O images which provide a clear representation of fibrous cap using high magnification (20x). In each fibroatheroma we measured the cap thickness in ten regions along the length of the cap. We used the cap thickness in the thinnest region for our analyses.

Luminal endothelial cell coverage was assessed by CD31 immunostaining on acetone-fixed cryosections. The cryosections were incubated with a 1:50 dilution of anti-CD31 antibody (Santa Cruz Biotech) overnight at 4\(^\circ\)C, washed with PBS twice and treated with a 1:200 dilution of an Alexa Flour 594 labeled secondary antibody (Invitrogen) for 1 hour at room temperature. Following four washes with PBS the samples were mounted in DAPI containing mounting
medium and imaged using fluorescence microscopy. CD31-positive areas indicating functional endothelium are expressed as percent of the periphery of the lumen.

**Immunohistochemistry for MMP-2**

Cryosections from representative arterial subsegments were fixed in acetone for 10 minutes prior to staining. Primary antibodies for MMP-2 (Santa Cruz Biotechnology) were diluted 1:200 in PBS containing 1% BSA, applied to slides, and incubated in a humid chamber overnight at 4°C. Secondary antibody staining was performed using the Envision-HRP kit (DakoCytomation, Carpinteria, CA) according to the manufacturer’s instructions. A DAB substrate (DakoCytomation) was used for detection of the HRP conjugate. The samples were counterstained in Mayer’s hematoxylin for 3 min, washed with tap water, and mounted in aqueous mounting medium (DakoCytomation).

**Immunofluorescent staining for CD45, MMP-2 and cathepsin S**

Cryosections from representative arterial subsegments were fixed in acetone at -20°C for 10 minutes prior to staining. Primary antibodies for MMP-2 (Santa Cruz Biotechnology), Cathepsin-S (Santa Cruz Biotechnology) or CD45 (Dako) were diluted 1:200 in PBS containing 1% BSA, applied to slides, and incubated in a humid chamber overnight at 4°C. The sections were washed with PBS twice, and secondary antibodies labeled with AlexaFluor-488 or -495 (Invitrogen) were applied at 1:500 dilution in PBS with BSA. After two hours of incubation at room temperature, the samples were washed extensively with PBS and then coverslipped with a DAPI-containing mounting media (Vector Laboratories). The sections were then imaged using an epifluorescent scope, and image processing/quantification was performed using Metamorph software (Molecular Devices, Inc.).
Messenger RNA analysis

Levels of mRNAs in the intima that encode selected matrix-degrading elastolytic proteases implicated in plaque remodeling (i.e. MMP-2, -9, 12, cathepsins K, L, S) and their inhibitors [i.e. tissue inhibitors of MMPs (TIMP)-1, -2, and cystatin C] were measured by real-time RT-PCR. The adventitia and surrounding heart tissue were removed from the cryosections by dissection under a dissecting microscope. The mRNA was isolated from the intima and media of cryosections of the arterial subsegments using the RNeasy Mini Protect mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. PCR was performed using a SYBR green master mix and an Applied Biosystems 7900HT Sequence Detection System. Cycle conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 94°C and 1 min at 60°C. Target gene mRNA levels were normalized to the “housekeeping” (i.e. GAPDH) mRNA level in each extract. The primers used in the PCR analysis are presented in Supplemental Table 1S.

In-situ zymography for MMPs and cathepsins

The protein activity and localization of MMPs and cathepsins was measured by in-situ zymography (ISZ), which can visualize enzymatic activity of different origin by optimizing experimental conditions (pH, specific inhibitors). We determined elastolytic activity in-situ in 8 µm thick, unfixed cryostat sections from the arterial subsegments of interest using elastin conjugated with quenched fluorescein, which requires cleavage by elastolytic enzymes to become fluorescent, as a substrate (DQ-elastin; Molecular Probes, Eugene, OR). Briefly, we mixed DQ-elastin (1 mg/ml) in a pH 5.5 buffer optimized for cysteine proteases with 1% low-melting-temperature agarose (Invitrogen), then added 20 µl of the mixture on top of each section, coverslipped, and let gel at 4°C. The contribution of metalloproteinases to elastolytic
activity was inhibited by 20 mM EDTA. MMPs activity was determined in an E64d-containing pH-7.4 buffer with or without EDTA (10 mM). Cysteine protease activity was determined using an EDTA-containing pH 5.5 buffer with or without E64d (20 µM). After 48 hours incubation at 37°C, sections were examined under a fluorescent microscope with all images captured under the same microscope settings and shutter conditions. The elastic lamina in the media showed auto-fluorescence under these conditions, providing a landmark for orientation of each section. Elastolytic activity-dependent fluorescence was measured as the percentage of intimal area, as previously described, by using the same threshold for all images. All ISZ images were obtained using the same original magnification (×100) and shutter speed. Autofluorescence of the IEL and media were excluded from the analyses. All ISZ experiments were done in duplicate.

**Histopathologic classification of lesions at follow-up**

Based on histopathologic characteristics, including IM, presence and thickness of fibrous cap in high magnification oil-red-O staining, the lesions were classified into three categories representing distinct stages of the natural history of atherosclerosis, using a modification of the classification proposed by Virmani et al. (i) Minimal lesions, defined as lesions with IM<0.15, were characterized by minimal depositions of lipids and inflammatory cells into the intima. The IM<0.15 threshold for the characterization of minimal lesions represented the lower quartile of the distribution of all the IM values in all analyzed sub-segments. More advanced lesions with IM≥0.15 were characterized as either intermediate lesions or thin cap fibroatheromata on the basis of the presence of a fibrous cap. (ii) Intermediate lesions, defined as lesions with IM≥0.15 without evidence of fibrous cap. (iii) Thin fibrous cap atheromata were characterized by a thin fibrous cap (minimal cap thickness
<65µm) overlying a lipid core.

Due to the intensely hyperlipidemic nature of the experimental model we did not observe fibrous lesions at week 30.

**IEL integrity**

The integrity of the subintimal internal elastic lamina (IEL) was assessed in Verhoeff’s elastin-stained sections and classified into the following four categories: grade 0 with intact, well-organized IEL; grade 1 with a few breaks in the IEL; grade 2 with many breaks in the IEL but having an intact media; grade 3 with severe fragmentation of the IEL associated with fragmentation of the underlying media.

**Assessment of the remodeling behavior of the subsegments of interest**

The nature of the arterial remodeling response to plaque growth in each arterial subsegment of interest was assessed by comparing the local remodeling behavior of each individual subsegment with the global remodeling response of the entire artery, which was used as reference. The EEM and lumen areas of all the IVUS cross-sections along each reconstructed artery were measured at follow-up (week 30), and plotted against the corresponding intima-media areas. The global reference of the entire reconstructed artery was determined by the linear regression line and its 95% prediction band in the EEM area vs. intima-media area plot and the lumen area vs. intima-media area plot. The EEM and lumen area of each individual subsegment of interest were then identified within the corresponding plots, and three local remodeling patterns were defined: (a) excessive expansive remodeling if the EEM and lumen area of the subsegment of interest was above the upper limit of the 95% prediction band of the entire artery remodeling behavior, (b) compensatory expansive remodeling if the EEM and lumen area of the subsegment of interest was within the 95% prediction band, and (c)
constrictive remodeling if the EEM and lumen area of the subsegment of interest was below the lower limit of the 95% prediction band.
Supplemental Study limitations

The arterial subsegments we investigated were not randomly selected – they were identified a priori based on their ESS maps. We did take care to include subsegments across a spectrum of baseline ESS magnitudes but could still have introduced selection bias in our samples especially as we were dealing with a restricted number of animals. Like all studies use of a larger number of animals at multiple times would have been beneficial if feasible. The power of the study increased, however, by investigating multiple subsegments in each coronary artery (average 5 subsegments per artery).

ESS was averaged within a 3-mm-long arterial subsegment, and was represented by a single histologic section derived from the middle of that subsegment. We assumed that the histologic section matched the ESS-derived subsegment by selecting relatively short subsegments of interest, which exhibit homogeneity of hemodynamic and histopathologic characteristics along their length.

The fibrous cap thickness was measured using oil-red-O stained sections. While this is the standard method for assessing fibrous cap thickness in human arterial sections, it does not provide simultaneous staining of both the fibrous and lipid components of the plaque. In our analyses we assumed that prominent staining for lipids with oil-red-O dye correlated with a loss of fibrous components in this region of the arterial section.

The entrance velocity profile was not measured by our methodology, because all possible entrance velocity profiles converge to a single profile within about 3 mm of the origin. The inlet velocity was assumed to be uniform, flow was assumed to be established after an entrance length of 3mm, and flow was ignored for the first 3 mm. The distortions introduced by these assumptions were insignificant at the Reynolds numbers observed in this study.²
### Supplemental Table 1S. Primer sequences of matrix-degrading proteases and their inhibitors

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>GAPDH</td>
<td>ACCCAGAAGACTGTGGATGG</td>
<td>TTGAGCTCAGGGATGACCTT</td>
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<tr>
<td>MMP-2</td>
<td>GACGGAGAGGCTGACATCAT</td>
<td>CCATACTTCACACGCACCAC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>ATGTTGGGCTACGTGACCTTC</td>
<td>CTCCCCCTTCTCTTGTCTCTC</td>
</tr>
<tr>
<td>MMP-12</td>
<td>CTGGACATGATGCAAAACC</td>
<td>AAAGCTTTCTGGATGGGTA</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>GCTCCCTGGAAACAGTCTGAG</td>
<td>GGTCTGTCCACAAGCAGTGA</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>GACTCCCGGGAACGACATCTA</td>
<td>CAGAGCGTGATGTCACATCTT</td>
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<td>Cathepsin K</td>
<td>CGCAGTAATGACACCCCTTT</td>
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<td>Cathepsin L</td>
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<td>Cathepsin S</td>
<td>ACAGTGCGCAGCTCGATTTTT</td>
<td>AACCTGGTGATGCTCAAC</td>
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<tr>
<td>Cystatin C</td>
<td>CGAGTACAACAAAGCGAGCA</td>
<td>AGGGACAGTTGTCCAGGTG</td>
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Supplemental Table 2S. Association of baseline ESS and total cholesterol with the mRNA expression of matrix-degrading enzymes and their inhibitors. B±SE represent the linear regression coefficient and the standard error. Standard errors are adjusted for clustering of segments with animals.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>ESS</th>
<th>p</th>
<th>Total Cholesterol</th>
<th>p</th>
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<td>MMP-2</td>
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<td>MMP-9</td>
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<td>0.022</td>
<td>0.004±0.001</td>
<td>0.004</td>
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<tr>
<td>MMP-12</td>
<td>-0.003±0.004</td>
<td>0.37</td>
<td>0.00003±0.00006</td>
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<td>TIMP-1</td>
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<td>TIMP-2</td>
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<td>0.001±0.0003</td>
<td>0.007</td>
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<td>MMP/TIMP</td>
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<td>Cathepsin K</td>
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<td>Cathepsin L</td>
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<td>Cathepsin S</td>
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<td>0.001</td>
<td>0.001±0.0005</td>
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<td>Cystatin C</td>
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<td>Total cathepsins/Cystatin C</td>
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<td>0.001±0.0007</td>
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Supplemental Table 3S. Distribution of the three types of remodeling pattern along the four grades of IEL fragmentation.

<table>
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<th>IEL Grade</th>
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<td><strong>Total</strong></td>
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<td><strong>142</strong></td>
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<tr>
<td><strong>Total</strong></td>
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<tr>
<td>3</td>
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<tr>
<td><strong>Total</strong></td>
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p=0.005 adjusted for the clustering of arteries within animals
Supplemental Figures and Figure Legends

**Supplemental Figure 1S.** Lesions with low baseline ESS exhibited higher lumen, external elastic membrane (EEM), and plaque volume at follow-up compared to lesions with higher baseline ESS.
Supplemental Figure 2S. Frequency distribution of atheromata with thin fibrous cap at week 30 across different ESS levels at week 23.
Supplemental Figure 3S. Frequency distribution of minimal lesions, intermediate lesions and thin-capped atheromata across the intima-to-media ratio range. Intermediate lesions and thin-capped atheromata with similar intima-to-media ratio between 0.3 and 0.9 (n=52) were selected and classified as atheromata without fibrous cap (n=32) or small atheromata with thin fibrous cap (n=20).
Supplemental References

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Identification of arterial subsegments of interest at baseline and follow-up

To investigate the effect of ESS on mRNA and protein expression of matrix degrading enzymes, ESS was calculated on the lumen surface of the reconstructed coronary arteries at baseline (week 23) and correlated with the mRNA and protein expression at follow-up (week 30). Arterial subsegments of interest 3 mm in length were selected on the basis of different levels of baseline ESS magnitude. The subsegments exhibited varied 3D geometry from very straight to curved sections. To be considered in the analysis, each subsegment of interest was required to be free of apparent atherosclerotic plaque at baseline, defined by IVUS as maximum intima and media thickness $\leq 0.5$ mm, and to have a surface area of similar ESS $\geq 30\%$ of the total surface area of the subsegment.\textsuperscript{2,5} We focused on subsegments free of apparent atherosclerosis by IVUS at baseline (week 23) because this allowed us to study the role of ESS in the initiation and progression of coronary atherosclerosis through multiple natural history stages culminating in high-risk plaque. To reduce the impact of local flow disturbances on our results, subsegments free of side branches and located at least 1 mm away from adjacent side branches were selected.\textsuperscript{2}

The subsegments of interest were located in the preserved coronary arteries.\textsuperscript{2} To aid in locating each subsegment, several major and readily visible side branches were identified at baseline and follow-up on the ESS and wall thickness maps. The same side branches were also identified on the harvested arteries by utilizing magnifying lenses (2x). Using these branches as landmarks the exact location of each subsegment of interest was then identified on the preserved coronary arteries. The middle portion of the frozen subsegments of interest was cryosectioned at 7 \( \mu \)m thickness for histopathologic analysis, mRNA isolation and \textit{in-situ} zymography (ISZ). Endothelial shear stress which was averaged within a 3 mm long arterial subsegment was represented by a single histologic section derived from the middle portion of that subsegment.
However, selection of relatively short subsegments of interest, which exhibit homogeneity of hemodynamic and histopathologic characteristics along their length\textsuperscript{8}, permitted accurate registration of the local ESS environment with the histopathologic findings.

**Histopathology**

The intima-to-media ratio (IM) was measured in Verhoeff’s elastin-stained cryosections by computerized planimetry. Lipid deposition and inflammatory cell infiltration were measured in oil red O- and CD45-stained cryosections. Immunostaining for CD45 used a monoclonal antibody (1:50, clone 2A5, BD Biosciences Inc., San Jose, CA) against pig CD45 leukocyte common antigen. Immunostaining for the class II histocompatibility complex (MHC-II) molecules (1:90, murine MHC class II, Pharmingen, San Diego, CA) provided an assessment of inflammatory cells activation in the atherosclerotic intima; parallel immunostaining for CD45 and MHC-II was used in n=56 available subsegments to evaluate the percentage of activated inflammatory cells.\textsuperscript{9,10} Oil red O-, CD45- and MHC-II positive intimal areas were assessed with a computer-assisted color-gated technique and are presented as percent of the intima.

Fibrous cap thickness was measured in Oil-red-O images which provide a clear representation of fibrous cap using high magnification (20x). In each fibroatheroma we measured the cap thickness in ten regions along the length of the cap. We used the cap thickness in the thinnest region for our analyses.

Luminal endothelial cell coverage was assessed by CD31 immunostaining on acetone-fixed cryosections. The cryosections were incubated with a 1:50 dilution of anti-CD31 antibody (Santa Cruz Biotech) overnight at 4°C, washed with PBS twice and treated with a 1:200 dilution of an Alexa Flour 594 labeled secondary antibody (Invitrogen) for 1 hour at room temperature. Following four washes with PBS the samples were mounted in DAPI containing mounting
medium and imaged using fluorescence microscopy. CD31-positive areas indicating functional endothelium are expressed as percent of the periphery of the lumen.

**Immunohistochemistry for MMP-2**

Cryosections from representative arterial subsegments were fixed in acetone for 10 minutes prior to staining. Primary antibodies for MMP-2 (Santa Cruz Biotechnology) were diluted 1:200 in PBS containing 1% BSA, applied to slides, and incubated in a humid chamber overnight at 4°C. Secondary antibody staining was performed using the Envision-HP kit (DakoCytomation, Carpinteria, CA) according to the manufacturer’s instructions. A DAB substrate (DakoCytomation) was used for detection of the HRP conjugate. The samples were counterstained in Mayer’s hematoxylin for 3 min, washed with tap water, and mounted in aqueous mounting medium (DakoCytomation).

**Immunofluorescent staining for CD45, MMP-2 and cathepsin S**

Cryosections from representative arterial subsegments were fixed in acetone at -20°C for 10 minutes prior to staining. Primary antibodies for MMP-2 (Santa Cruz Biotechnology), Cathepsin-S (Santa Cruz Biotechnology) or CD45 (Dako) were diluted 1:200 in PBS containing 1% BSA, applied to slides, and incubated in a humid chamber overnight at 4°C. The sections were washed with PBS twice, and secondary antibodies labeled with AlexaFluor-488 or -495 (Invitrogen) were applied at 1:500 dilution in PBS with BSA. After two hours of incubation at room temperature, the samples were washed extensively with PBS and then coverslipped with a DAPI-containing mounting media (Vector Laboratories). The sections were then imaged using an epifluorescent scope, and image processing/quantification was performed using Metamorph software (Molecular Devices, Inc.).
Messenger RNA analysis

Levels of mRNAs in the intima that encode selected matrix-degrading elastolytic proteases implicated in plaque remodeling (i.e. MMP-2, -9, 12, cathepsins K, L, S) and their inhibitors [i.e. tissue inhibitors of MMPs (TIMP)-1, -2, and cystatin C] were measured by real-time RT-PCR. The adventitia and surrounding heart tissue were removed from the cryosections by dissection under a dissecting microscope. The mRNA was isolated from the intima and media of cryosections of the arterial subsegments using the RNeasy Mini Protect mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. PCR was performed using a SYBR green master mix and an Applied Biosystems 7900HT Sequence Detection System. Cycle conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 94°C and 1 min at 60°C. Target gene mRNA levels were normalized to the “housekeeping” (i.e. GAPDH) mRNA level in each extract. The primers used in the PCR analysis are presented in Supplemetal Table 1S.

In-situ zymography for MMPs and cathepsins

The protein activity and localization of MMPs and cathepsins was measured by in-situ zymography (ISZ), which can visualize enzymatic activity of different origin by optimizing experimental conditions (pH, specific inhibitors). We determined elastolytic activity in-situ in 8 µm thick, unfixed cryostat sections from the arterial subsegments of interest using elastin conjugated with quenched fluorescein, which requires cleavage by elastolytic enzymes to become fluorescent, as a substrate (DQ-elastin; Molecular Probes, Eugene, OR). Briefly, we mixed DQ-elastin (1 mg/ml) in a pH 5.5 buffer optimized for cysteine proteases 1:10 with 1% low-melting-temperature agarose (Invitrogen), then added 20 µl of the mixture on top of each section, coverslipped, and let gel at 4°C. The contribution of metalloproteininas to elastolytic
activity was inhibited by 20 mM EDTA. MMPs activity was determined in an E64d-containing pH-7.4 buffer with or without EDTA (10 mM). Cysteine protease activity was determined using an EDTA-containing pH 5.5 buffer with or without E64d (20 µM). After 48 hours incubation at 37°C, sections were examined under a fluorescent microscope with all images captured under the same microscope settings and shutter conditions. The elastic lamina in the media showed auto-fluorescence under these conditions, providing a landmark for orientation of each section. Elastolytic activity-dependent fluorescence was measured as the percentage of intimal area, as previously described,\textsuperscript{13,14} by using the same threshold for all images. All ISZ images were obtained using the same original magnification (×100) and shutter speed. Autofluorescence of the IEL and media were excluded from the analyses. All ISZ experiments were done in duplicate.

**Histopathologic classification of lesions at follow-up**

Based on histopathologic characteristics, including IM, presence and thickness of fibrous cap in high magnification oil-red-O staining, the lesions were classified into three categories representing distinct stages of the natural history of atherosclerosis, using a modification of the classification proposed by Virmani et al.\textsuperscript{2,15} 

(i) Minimal lesions, defined as lesions with IM<0.15, were characterized by minimal depositions of lipids and inflammatory cells into the intima. The IM<0.15 threshold for the characterization of minimal lesions represented the lower quartile of the distribution of all the IM values in all analyzed sub-segments. More advanced lesions with IM≥0.15 were characterized as either intermediate lesions or thin cap fibroatheromata on the basis of the presence of a fibrous cap.

(ii) Intermediate lesions, defined as lesions with IM≥0.15 without evidence of fibrous cap.

(iii) Thin fibrous cap atheromata were characterized by a thin fibrous cap (minimal cap thickness
<65µm) overlying a lipid core.

Due to the intensely hyperlipidemic nature of the experimental model we did not observe fibrous lesions at week 30.

**IEL integrity**

The integrity of the subintimal internal elastic lamina (IEL) was assessed in Verhoeff’s elastin-stained sections and classified into the following four categories\(^2\)\(^{,12}\): grade 0 with intact, well-organized IEL; grade 1 with a few breaks in the IEL; grade 2 with many breaks in the IEL but having an intact media; grade 3 with severe fragmentation of the IEL associated with fragmentation of the underlying media.

**Assessment of the remodeling behavior of the subsegments of interest**

The nature of the arterial remodeling response to plaque growth in each arterial subsegment of interest was assessed by comparing the local remodeling behavior of each individual subsegment with the global remodeling response of the entire artery, which was used as reference.\(^2\)\(^{,3}\) The EEM and lumen areas of all the IVUS cross-sections along each reconstructed artery were measured at follow-up (week 30), and plotted against the corresponding intima-media areas. The global reference of the entire reconstructed artery was determined by the linear regression line and its 95% prediction band in the EEM area vs. intima-media area plot and the lumen area vs. intima-media area plot. The EEM and lumen area of each individual subsegment of interest were then identified within the corresponding plots, and three local remodeling patterns were defined: (a) excessive expansive remodeling if the EEM and lumen area of the subsegment of interest was above the upper limit of the 95% prediction band of the entire artery remodeling behavior, (b) compensatory expansive remodeling if the EEM and lumen area of the subsegment of interest was within the 95% prediction band, and (c)
constrictive remodeling if the EEM and lumen area of the subsegment of interest was below the lower limit of the 95% prediction band.
Supplemental Study limitations

The arterial subsegments we investigated were not randomly selected – they were identified a priori based on their ESS maps. We did take care to include subsegments across a spectrum of baseline ESS magnitudes but could still have introduced selection bias in our samples especially as we were dealing with a restricted number of animals. Like all studies use of a larger number of animals at multiple times would have been beneficial if feasible. The power of the study increased, however, by investigating multiple subsegments in each coronary artery (average 5 subsegments per artery).

ESS was averaged within a 3-mm-long arterial subsegment, and was represented by a single histologic section derived from the middle of that subsegment. We assumed that the histologic section matched the ESS-derived subsegment by selecting relatively short subsegments of interest, which exhibit homogeneity of hemodynamic and histopathologic characteristics along their length.

The fibrous cap thickness was measured using oil-red-O stained sections. While this is the standard method for assessing fibrous cap thickness in human arterial sections, it does not provide simultaneous staining of both the fibrous and lipid components of the plaque. In our analyses we assumed that prominent staining for lipids with oil-red-O dye correlated with a loss of fibrous components in this region of the arterial section.

The entrance velocity profile was not measured by our methodology, because all possible entrance velocity profiles converge to a single profile within about 3 mm of the origin. The inlet velocity was assumed to be uniform, flow was assumed to be established after an entrance length of 3mm, and flow was ignored for the first 3 mm. The distortions introduced by these assumptions were insignificant at the Reynolds numbers observed in this study.²
**Supplemental Tables**

**Supplemental Table 1S.** Primer sequences of matrix-degrading proteases and their inhibitors

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<thead>
<tr>
<th>mRNA</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>ACCCAGAAGACTGTGGATGG</td>
<td>TTGAGCTCAGGGATGACCTT</td>
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<tr>
<td>MMP-2</td>
<td>GACGGAGAGGCTGACATCAT</td>
<td>CCATACTTCACACGCACCAC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>ATGTGGGCTACGTCACCTTC</td>
<td>CTCCCCCTTCTCCTTGTCCTC</td>
</tr>
<tr>
<td>MMP-12</td>
<td>CTGGACATGATGCACAAACC</td>
<td>AAAGCTTTCTGGATGGCGTA</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>GCTCCCTGGAAACAGTCTGAG</td>
<td>GGTCCTGTCACAAAGCAGTGA</td>
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<tr>
<td>TIMP-2</td>
<td>GACTCCGGGAACGACATCTA</td>
<td>CAGAGCGTGATGTGCACTCTT</td>
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<tr>
<td>Cathepsin K</td>
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<tr>
<td>Cathepsin L</td>
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<tr>
<td>Cathepsin S</td>
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<tr>
<td>Cystatin C</td>
<td>CGAGTACAACAAAGCGAGCA</td>
<td>AGGGACAGTTGTCCAGGT TG</td>
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### Supplemental Table 2S

Association of baseline ESS and total cholesterol with the mRNA expression of matrix-degrading enzymes and their inhibitors. B±SE represent the linear regression coefficient and the standard error. Standard errors are adjusted for clustering of segments with animals.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>ESS</th>
<th>p</th>
<th>Total Cholesterol</th>
<th>p</th>
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<tr>
<td>MMP-2</td>
<td>-0.017±0.005</td>
<td>0.01</td>
<td>0.00015±0.000057</td>
<td>&lt;0.0001</td>
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<tr>
<td>MMP-9</td>
<td>-0.54±0.19</td>
<td>0.022</td>
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<tr>
<td>MMP-12</td>
<td>-0.003±0.004</td>
<td>0.37</td>
<td>0.00003±0.00006</td>
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<td>TIMP-1</td>
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<td>0.00036±0.0002</td>
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<tr>
<td>TIMP-2</td>
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<td>0.002</td>
<td>0.001±0.0003</td>
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<tr>
<td>MMP/TIMP</td>
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<tr>
<td>Cathepsin K</td>
<td>-0.19±0.12</td>
<td>0.16</td>
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<tr>
<td>Cathepsin L</td>
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<td>Cathepsin S</td>
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<td>0.001±0.0005</td>
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<td>Cystatin C</td>
<td>-0.69±0.13</td>
<td>&lt;0.0001</td>
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<td>Total cathepsins/Cystatin C</td>
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<td>0.4</td>
<td>0.001±0.0007</td>
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Supplemental Table 3S. Distribution of the three types of remodeling pattern along the four grades of IEL fragmentation.

<table>
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<tr>
<th>IEL Grade</th>
<th>Remodeling Pattern</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>Constrictive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Compensatory expansive</td>
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</tr>
<tr>
<td></td>
<td>Excessive expansive</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
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</tr>
<tr>
<td>2</td>
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<td>32</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>72</td>
</tr>
</tbody>
</table>

p=0.005 adjusted for the clustering of arteries within animals
Supplemental Figures and Figure Legends

**Supplemental Figure 1S.** Lesions with low baseline ESS exhibited higher lumen, external elastic membrane (EEM), and plaque volume at follow-up compared to lesions with higher baseline ESS.
Supplemental Figure 2S. Frequency distribution of atheromata with thin fibrous cap at week 30 across different ESS levels at week 23.
**Supplemental Figure 3S.** Frequency distribution of minimal lesions, intermediate lesions and thin-capped atheromata across the intima-to-media ratio range. Intermediate lesions and thin-capped atheromata with similar intima-to-media ratio between 0.3 and 0.9 (n=52) were selected and classified as atheromata without fibrous cap (n=32) or small atheromata with thin fibrous cap (n=20).
Supplemental References