Thin-Capped Atheromata With Reduced Collagen Content in Pigs Develop in Coronary Arterial Regions Exposed to Persistently Low Endothelial Shear Stress


Objective—The mechanisms promoting the focal formation of rupture-prone coronary plaques in vivo remain incompletely understood. This study tested the hypothesis that coronary regions exposed to low endothelial shear stress (ESS) favor subsequent development of collagen-poor, thin-capped plaques.

Approach and Results—Coronary angiography and 3-vessel intravascular ultrasound were serially performed at 5 consecutive time points in vivo in 5 diabetic, hypercholesterolemic pigs. ESS was calculated along the course of each artery with computational fluid dynamics at all 5 time points. At follow-up, 184 arterial segments with previously identified in vivo ESS underwent histopathologic analysis. Compared with other plaque types, eccentric thin-capped atheromata developed more in segments that experienced lower ESS during their evolution. Compared with lesions with higher preceding ESS, segments persistently exposed to low ESS (<1.2 Pa) exhibited reduced intimal smooth muscle cell content; marked intimal smooth muscle cell phenotypic modulation; attenuated procollagen-I gene expression; increased gene and protein expression of the interstitial collagenases matrix-metalloproteinase-1, -8, -13, and -14; increased collagenolytic activity; reduced collagen content; and marked thinning of the fibrous cap.

Conclusions—Eccentric thin-capped atheromata, lesions particularly prone to rupture, form more frequently in coronary regions exposed to low ESS throughout their evolution. By promoting an imbalance of attenuated synthesis and augmented collagen breakdown, low ESS favors the focal evolution of early lesions toward plaques with reduced collagen content and thin fibrous caps—2 critical determinants of coronary plaque vulnerability. (Arterioscler Thromb Vasc Biol. 2013;33:1494-1504.)

Key Words: atherosclerosis ■ collagen ■ endothelial shear stress ■ metalloproteinases ■ natural history

Although advanced plaques that rupture and trigger most acute coronary events have well-defined morphology and histological characteristics, the preceding local environment and the mechanisms that promote the evolution of early lesions toward rupture-prone thin-capped atheroma (TCA) remain largely unknown. Low regional endothelial shear stress (ESS) promotes focal plaque development and progression and critically influences plaque vulnerability. Previous studies have established the mechanistic link between low ESS and atherogenesis in vitro, but the mechanisms whereby low ESS favors the localization of rupture-prone coronary lesions in vivo remain poorly understood. This study explored, in particular, 2 issues with important clinical implications not addressed in prior studies: the in vivo effect of ESS environment on (1) local collagen metabolism and (2) smooth muscle cell (SMC) phenotype.

Interstitial collagen strengthens the fibrous cap and likely enhances its resistance to rupture. Members of the matrix-metalloproteinase (MMP) family with collagenase activity, including MMP-1, -8, -13, and the activator of MMP collagenases, MMP-14, weaken the plaque by degrading collagen fibers. Low ESS associates with increased expression of extracellular matrix–degrading enzymes ex vivo and with the formation of collagen-poor carotid lesions in mice. Previous studies, however, used isolated cells or examined only 2 time points to assess natural
history—approaches which may not reflect the complexity and dynamic nature of atherosclerotic disease.\textsuperscript{9,20} Although low ESS induces elastolytic enzymes in coronary atheroma,\textsuperscript{21} the in vivo effect of ESS on the regional expression of collagen-degrading enzymes—and thereby on the local control of collagen content—throughout the progression of individual coronary lesions remains unknown.

The content and synthetic capacity of collagen-producing SMCs contribute centrally to regulating plaque collagen turnover.\textsuperscript{1,4,22} Abundance of intimal SMCs favors plaque quiescence, whereas the relative absence of SMCs\textsuperscript{2,3} and extensive phenotypic modulation\textsuperscript{23} both characterize fatally disrupted human coronary plaques. Low ESS associates with SMC phenotypic modulation\textsuperscript{24} and apoptosis in vitro.\textsuperscript{25} The in vivo role of ESS in regulating SMC content and functions and their implications concerning the heterogeneity of coronary plaque manifestations have undergone only minimal exploration.

This study hypothesized that coronary arterial regions exposed to low ESS more commonly progress toward eccentric TCA (eTCA) morphology—a lesion type implicated in plaque rupture and thrombosis in humans.\textsuperscript{1,2} Testing this hypothesis involved in vivo serial profiling of ESS at 5 consecutive time points over the course of plaque development, followed by histological analysis of the same lesions with previously identified in vivo ESS. Considering the critical impact of collagen on the structural integrity of coronary atheromata,\textsuperscript{3,14–17} a secondary goal evaluated the association of ESS with the local control of plaque collagen content. We, therefore, assessed the amount and phenotype of SMCs, and the expression and activity of MMP collagenases in lesions that originated from, and evolved through, substantially different ESS environments. We analyzed diabetic, hyperlipidemic pigs capable of developing plaques very similar to those observed in humans.\textsuperscript{10,21,26}

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Dynamic Course of Local ESS in Individual Arterial Segments

Local ESS exhibited substantial changes over time (weeks 4–36) throughout the progression of plaque in individual 3-mm-long arterial segments (n=184). A substantial proportion of segments with low ESS, ranging between 12.4% and 47.3% over time, changed to higher ESS at the next time point. Similarly, a proportion ranging between 14% and 44.3% of segments with higher ESS at each time point changed to low ESS at the following time point (Figure 1 in the online-only Data Supplement). These dynamic changes of ESS are related to variable responses of the vessel wall and lumen dimensions throughout the course of plaque evolution in each segment (Figures II and III in the online-only Data Supplement).

Because low ESS associates in a time- and level-dependent manner with the rate of subsequent plaque progression\textsuperscript{4,8} and with the magnitude of high-risk plaque characteristics,\textsuperscript{10,21} we identified the minority of segments (n=75; 41%) that remained in persistently low ESS throughout their evolution, defined by time-averaged ESS<1.2 Pa through all 5 time points (weeks 4–36). All our analyses compared these segments by histology at follow-up (week 36) with all other segments that developed in a higher ESS environment over time, defined by time-averaged ESS\geq1.2 Pa (n=109; 59%).

eTCA Develop in Regions With Persistently Low ESS

Segments were classified at follow-up as segments with minimal lesion (n=10; 6%), with intermediate lesion (n=47; 25%), concentric TCA (n=96; 52%), and eTCA plaque morphology (n=31; 17%; Figure 1A). We focused on the natural history of eTCA as the highest risk plaque type among all heterogeneous segments because thin-capped plaques with eccentric morphology seem particularly prone to rupture and cause acute coronary events in humans.\textsuperscript{27,28} In line with these human data,\textsuperscript{27,28} we found that eTCA had thinner fibrous caps, more frequently contained true necrotic cores, and had larger necrotic core areas compared with concentric TCA (Figure 1B–1E).

We assessed the study’s primary end point—the relation between the preceding local ESS over time and subsequent development of eTCA at follow-up—using a dual approach: (1) a plaque type-based approach, comparing the preceding ESS in eTCA with all other plaque types; and (2) an ESS-based approach, prospectively comparing the proportion of segments with persistently low ESS with segments with higher ESS that subsequently progressed to eTCA.

eTCA arose more frequently from persistently low-ESS segments than from higher ESS segments (74% versus 26% of all eTCA, respectively). eTCA originated from persistently low-ESS segments more frequently (74% of eTCA), compared with all other plaque types that originated from persistently low-ESS segments (34% of non-eTCA lesions; \(P<0.0001\); Figure 2A). The preceding ESS in eTCA was lower at all 5 time points compared with all other, non-eTCA plaque types (Figure 2B and 2C). The significant decrease of ESS at week 16 in both eTCA and non-eTCA segments is associated with substantial increase of vessel and lumen dimensions at this time point (Figure IV in the online-only Data Supplement). Segments with eTCA plaque morphology at follow-up had a higher time-averaged ESS eccentricity index and more commonly had an eccentric ESS pattern compared with segments with non-eTCA morphology (Figure 2D and 2E).

Persistently low-ESS segments subsequently resulted in eTCA more frequently than did higher ESS segments (31% versus 7%, respectively; \(P<0.0001\); Figure 2F). The incidence of eTCA morphology at follow-up was highest in segments with low ESS at all 5 individual time points (Figure 2G). The positive predictive value of persistently low ESS to identify subsequent eTCA morphology rose from 31% (Table II in the online-only Data Supplement) to 52% when low ESS at all 5 time points and eccentric ESS were both present (Figure 2H). The negative predictive value of persistently low ESS to predict subsequent eTCA morphology was even higher (92.7%) and it remained very high (89.2%) when the combination of low ESS at all 5 time points plus eccentric ESS was present (Figure 2H).
Reduced Intimal Collagen Content in Arterial Segments With Persistently Low ESS

Persistently low-ESS segments were not only larger at follow-up (Figure 3A) but also differed substantially in plaque composition. Persistently low-ESS segments versus higher ESS segments showed decreased intimal content of fibrillar collagen (Figure 3B and 3C). Time-averaged ESS as a continuous variable associated significantly with intimal collagen content ($r=0.60$; $P<0.0001$); for each decrease of time-averaged ESS by 1.0 Pa, intimal collagen content decreased by 17.9% (Figure 3D). Persistently low-ESS segments also showed reduced expression of procollagen type-I mRNA that encodes the main precursor molecule of fibrillar collagen type-I (Figure 3E), indicating that the reduced collagen content in low-ESS segments derives at least in part from decreased procollagen-I production. Transmission electron microscopy analysis documented few collagen fibers at the fibrous cap region of persistently low-ESS segments, relative to the abundant, well-organized collagen fibers in higher ESS segments (Figure 3F).

Reduced Content and Marked Phenotypic Modulation of Intimal SMCs in Arterial Segments With Persistently Low ESS

Persistently low-ESS segments showed decreased intimal SMC content compared with higher ESS segments (Figure 4A and 4B). The $\alpha$-actin–positive intimal area positively correlated to the collagen-stained area ($r=0.80$; $P=0.001$), a finding consistent with the function of SMCs as the main source of collagen in the atherosclerotic intima.

Quantitative analysis of apoptotic nuclei that colocalized with $\alpha$-actin–positive intimal areas in serial sections of selected segments demonstrated a 3-fold higher number of apoptotic cells in persistently low-ESS segments versus higher ESS segments (Figure 4C and 4D). Apoptosis of intimal SMCs in low-ESS segments was assessed directly by $\alpha$-actin immunofluorescent staining combined with terminal deoxynucleotidyl transferase dUTP nick end labeling staining (Figure 4E).

Persistently low-ESS segments versus higher ESS segments showed increased mRNA levels of platelet-derived growth factor (Figure 5A) and decreased mRNA levels of desmin and smoothelin, characteristic markers of unmodulated SMCs. The desmin-to-$\alpha$-actin ratio and the smoothelin-to-$\alpha$-actin ratio—indices previously used to quantify the extent of SMC phenotypic modulation—were <1 in all segments, but these ratios fell particularly in persistently low-ESS segments (Figure 5B), suggesting that the reduced mRNA expression of desmin and smoothelin relative to $\alpha$-actin observed in all segments was even more prominent in those with low ESS. Double immunofluorescent staining for $\alpha$-actin and desmin affirmed reduced desmin expression relative to $\alpha$-actin in the intima of persistently low-ESS segments (Figure 5C, top), suggesting the presence of modulated SMCs, whereas these 2 proteins showed greater colocalization in the intima of higher ESS segments (Figure 5C, bottom). Desmin and $\alpha$-actin colocalized in the tunica media, an arterial wall layer known to accommodate unmodulated, contractile SMCs.

Increased Expression and Activity of MMP Collagenases in Arterial Segments With Persistently Low ESS

Segments with persistently low ESS versus higher ESS had increased levels of mRNAs that encode MMP-1, MMP-13,
MMP-14, and tissue inhibitor of MMP (TIMP)-1 (Figure 6A), whereas TIMP-2 mRNA levels did not differ (not shown). Despite the parallel increase of the MMPs and one of their endogenous inhibitors (ie, TIMP-1), persistently low-ESS segments had increased MMP/TIMP mRNA ratios (Figure 6A). Consistent with our mRNA results, persistently low-ESS segments exhibited greater MMP-1, MMP-8, and MMP-13 protein expression than higher ESS segments (representative examples shown in Figure 6B). MMP-1, a major interstitial collagenase in humans, increased >3-fold in segments with persistently low ESS versus higher ESS, and its expression was inversely related to the time-averaged ESS (Figure 6C and 6D).

Persistently low-ESS segments showed more pronounced bright green fluorescence by in situ zymography than did higher ESS segments, indicating cleavage of the collagen substrate (Figure 7A). The enhanced collagenolytic activity localized with pronounced leukocyte infiltration and MMP-1 protein expression, as well as with reduced intimal collagen content and pronounced fibrous cap thinning (Figure 7A), consistent with the contribution of MMP-1 to collagen degradation in a low-ESS milieu. Quantitative analyses showed higher mRNA expression of monocyte chemoattractant protein-1, increased CD45-positive leukocyte content, and 3-fold greater collagenolytic activity in persistently low-ESS versus higher ESS segments (Figure 7B–7D). In addition to MMP-1, enhanced protein expression of MMP-8, MMP-13, and of the collagenase activator MMP-14 in low-ESS segments localized with regions of marked collagenolysis (Figure 7E). Addition of the metallo-enzyme inhibitor EDTA (20 mmol/L) abolished zymographic activity (Figure 7E), an indication that these MMPs contribute to collagenolytic activity.

Discussion

Although advanced plaques that rupture and provoke acute coronary thrombosis in humans have a well-characterized morphology, the mechanisms that promote the focal progression of early lesion to rupture-prone plaques in vivo remain poorly understood. Because all regions of the arterial tree experience similar exposure to traditional risk factors, such as dyslipidemia and hyperglycemia, we hypothesized that the preceding local hemodynamic environment influences decisively the clinically crucial segmental nature of lesions prone to rupture. To test our hypothesis, we performed a natural history study, uniquely designed with multiple

Figure 2. A, Eccentric thin-capped atheromata (eTCA) formed more frequently in segments with persistently low endothelial shear stress (ESS) over time (23 of 31 eTCA; 74%) than in higher ESS segments (8 of 31 eTCA; 26%; P<0.0001). In contrast, all other plaque types rarely derived from persistently low-ESS segments (52 of 153 non-eTCA lesions; 34%). Compared with all other plaque types, eTCA had lower levels of ESS (B) and were more frequently exposed to low ESS (<1.2 Pa; C) at all 5 time points throughout their progression (weeks 4–36). Segments that developed eTCA more frequently had eccentric ESS (D) and had a higher ESS Eccentricity Index (E) compared with segments that developed concentric thin-capped atheromata (cTCA). F, Segments with persistently low ESS resulted in eTCA more frequently (23 of 75 low-ESS segments; 30.7%) compared with higher ESS segments that resulted in eTCA (8 of 109 higher ESS segments; 7.3%; P<0.0001). G, Incidence of eTCA morphology at follow-up in relation to the number of individual time points with low ESS<1.2 Pa. H, Incidence of eTCA plaque morphology for segments with vs those without presence of persistently low ESS; low ESS at all 5 time points; and low ESS at all 5 time points plus eccentric ESS.
sessions of in vivo vascular profiling over the course of plaque development and with subsequent histopathology in atherosclerotic pigs, which develop lesions similar to human coronary atherosclerosis. Although local ESS displayed a dynamic course over time, regions with persistently low, eccentric ESS throughout their evolution tended to give rise to eTCA—a plaque type associated in humans with acute disruption and, prospectively, with adverse clinical events. Persistently low, eccentric ESS had a remarkably high negative predictive value (>90%) for prediction of eTCA development, suggesting that high-risk coronary plaque development is extremely uncommon in the absence of these local hemodynamic conditions. These novel observations link to evidence presented here concerning the local control of atheroma collagen, an extracellular matrix macromolecule that critically influences plaque stability. Regions of low ESS are associated with the formation of lesions with reduced SMC content and augmented expression and activity of collagenases, favoring attenuated synthesis and increased catabolism of collagen—an imbalance that likely contributes to the evolution of early lesions to collagen-poor TCA. Our findings thereby extend prior mechanistic studies that directly link low ESS to atherogenesis and plaque inflammation, and they support a critical role of low, eccentric ESS in the development of high-risk coronary plaque in vivo.

Dynamic Nature and Predictive Value of Local ESS

Previous investigations consistently associated low ESS with plaque progression and destabilization. In addition to ESS magnitude, circumferential ESS heterogeneity has also been linked to plaque instability. These previous studies were, nonetheless, limited because they defined high-risk plaque by intravascular ultrasound—a modality insufficient to assess plaque composition—or because they examined only 2 time points, interpreting local vascular behavior as a function of a single value of preceding ESS. Local ESS patterns, however, depend both on vascular geometric configurations and on atherosclerotic plaque-induced alterations of arterial geometry and wall remodeling, which may all change over time. This study overcame those fundamental limitations by uniquely combining serial in vivo profiling of local ESS in the entire coronary tree, from early plaque initiation to the development of advanced atheroma, with assessment of subsequent plaque morphology by histological examination—the ultimate standard for tissue characterization. Our findings advance the current appreciation of low ESS as a critical proatherogenic factor in 2 important ways. First, we demonstrate that local ESS in regions where plaque forms and progresses can vary substantially over time; these dynamic changes of local ESS result from variable contributions of the arterial wall’s remodeling response, and thereby from dynamic changes of vessel and lumen dimensions over the
course of local plaque progression (Figures II–IV in the online-only Data Supplement). Second, we show that a local environment of persistently low, eccentric ESS characterizes only a small proportion of developing lesions and correlates tightly with the focal formation of eTCA—a morphology associated in humans with rupture and fatal thrombotic events. Previous studies using a single snapshot of baseline ESS documented an exposure–response relation between the magnitude of low ESS and the level of high-risk plaque characteristics.10,21 Our present study now extends those findings over time by showing an exposure–response relation between the duration of exposure to low ESS and the propensity to high-risk plaque development; the longer an arterial regions experiences low ESS throughout its evolution, the more likely it will progress toward a rupture-prone eTCA. Novel evidence presented here, associating low ESS with the pathobiology of extracellular matrix metabolism, implicated in high-risk plaque formation, substantiates these observations.

Current imaging modalities have focused on the in vivo detection of advanced TCA before they rupture and thus become symptomatic.1 But identification of high-risk coronary plaques a step earlier in their natural history, that is prospective identification of early lesions before they progress toward rupture-prone TCA, remains a major clinical challenge. Low ESS, a lesion-related factor now measured in large-scale clinical studies,4 predicts subsequent plaque enlargement in humans.4,5 Our present experimental results advance these clinical observations and suggest that in vivo profiling of ESS magnitude and eccentricity might enhance the prediction not only of plaque enlargement but also of high-risk plaque formation early in its natural history. We show that persistently low ESS is a lesion-related characteristic conducive to eTCA development (with a positive predictive value of 30%), and that the combination of persistently low and eccentric ESS increased the positive predictive value to >50%.

An even more important finding of this study is that high-risk plaque is highly unlikely to develop in the absence of these local hemodynamic characteristics (with a negative predictive value >90%). Not all arterial segments with these characteristics developed eTCA, a finding that indicates that systemic and local factors not explored here also affect high-risk plaque development, likely including the magnitude of hypercholesterolemia,34 wall stress, disturbed flow,6 and strain.32

**Association of Low ESS With SMC Content and Phenotype**

This in vivo study also investigated the association of low ESS with the local control of plaque collagen. We found reduced content and more phenotypic modulation of intimal SMCs, the main collagen-producing cells in the atheroma, in coronary plaques previously exposed to low ESS. Vascular SMC phenotype can range from an unmodulated state with high content of contractile proteins to a modulated (so-called synthetic) state characterized by low content of contractile proteins and evidence of augmented protein synthesis.30 Late markers of unmodulated SMCs, such as smoothelin, desmin, or myosin, fall to a varying extent during the progression of human35 and pig atherosclerosis,31 typifying the transition of intimal SMCs to a more modulated state, while maintaining expression of α-actin.35 SMC modulation may promote plaque vulnerability,11 likely through the production of matrix-degrading enzymes.36 Our present findings add to the current appreciation of intimal SMC scarcity,22 SMC apoptosis,33,36 and profound SMC modulation,25,26 as features of unstable plaques by demonstrating a novel link between the preceding ESS milieu and the subsequent status of SMC content and character. Our
in vivo findings in a large-animal, human-like model of disease add to the results of previous in vitro experiments that mechanistically link low ESS to SMC apoptosis,\textsuperscript{25} and furnish 1 potential mechanism whereby low ESS may favor the formation of SMC-poor, high-risk lesions. Our finding of increased platelet-derived growth factor expression in regions with low ESS may be one of possible mechanisms underlying the marked phenotypic modulation of intimal SMCs. Platelet-derived growth factor is a growth factor that is upregulated by low shear stress in cell-culture studies\textsuperscript{24,39} and amplifies SMC modulation in the atheroma.\textsuperscript{39}

Local Regulation of Collagen Content Associates With the Preceding ESS
A dynamic balance between synthesis and enzymatic degradation actively regulates plaque collagen turnover,\textsuperscript{14} but the mechanisms responsible for the marked heterogeneity of collagen content along the atherosclerotic coronary vasculature have remained elusive. This study provides novel insight into the in vivo role of low ESS in the local regulation of collagen content and the focal formation of collagen-poor TCA. Modulated (synthetic) SMCs in low-ESS regions likely produced more collagen than the less modulated SMCs in higher ESS regions.\textsuperscript{30} We found, however, substantially reduced levels of mRNA that encodes type-I procollagen and decreased intimal collagen content in regions with persistently low ESS. This seemingly paradoxical finding might result from the profoundly reduced SMC content in low-ESS regions, which likely outweighed the enhanced collagen-producing capacity of the modulated, yet scarce, SMCs. In addition, despite favoring modulated SMC morphology, low ESS may functionally suppress collagen synthesis by enhancing interferon-\(\gamma\) and by decreasing transforming growth factor-\(\beta\), a potent promoter of collagen formation.\textsuperscript{40} Moreover, although collagenases in atheromata derive mainly from leukocytes,\textsuperscript{14} modulated SMCs also produce collagen-degrading MMP-8\textsuperscript{41} (Figure VIII in the online-only Data Supplement) that might, along with other collagenases, contribute to collagen breakdown and reduced collagen accumulation in low-ESS regions.

MMP collagenases participate decisively in the regulation of plaque collagen content.\textsuperscript{14–17} This study extends current knowledge by uniquely highlighting nonuniform expression of MMP collagenases in different coronary lesions that evolved through different local ESS environments. Regions with persistently low ESS showed increased expression of MMP-1, MMP-8, MMP-13, and MMP-14, preponderance over their endogenous inhibitors, and concomitant excess of metalloenzyme-mediated collagenolytic activity. The enhanced expression of monocyte chemoattractant protein-1 in low-ESS regions may promote leukocyte infiltration, likely

Figure 5. Relative mRNA levels of platelet-derived growth factor (PDGF) (A), desmin, smoothelin, and the desmin-to-\(\alpha\)-actin and smoothelin-to-\(\alpha\)-actin mRNA ratios (B) in persistently low-endothelial shear stress (ESS) vs higher ESS segments. C, Immunofluorescence for \(\alpha\)-actin (green; left), desmin (red; middle), and double staining for both antigens (merged; right) in segments with persistently low ESS (top) and higher ESS (bottom). Note the preponderance of green in the intima of the low-ESS segment (upper right), indicating the presence of desmin-negative, modulated smooth muscle cells (SMCs), and the orange color from the merged green and red in the intima of the higher ESS segment (lower right), indicating desmin-positive, unmodulated SMCs. Asterisks denote the lumen.
in concert with other chemoattractants. Increased expression of MMP collagenases, mainly by leukocytes, can initiate the proteolytic cleavage of interstitial collagens and set the stage for later steps of collagen’s catabolic cascade. Consequently, intense collagen digestion in inflamed lesions persistently exposed to low ESS likely drives the evolution of early lesions to advanced atheromata with reduced collagen content and marked fibrous cap thinning—both critical steps in rendering a plaque conducive to rupture.

Attenuation of proteolytic activity in rabbits yielded collagen-rich atheromata, despite the unmodulated phenotype of lesional SMCs, suggesting that the contribution of collagen degradation probably outweighs that of SMC-mediated synthesis to the regulation of plaque collagen content. In line with these previous experiments, in this study the net effect of decreased SMC content and augmented collagenolysis likely outweighed the ostensibly increased collagen-producing capacity of the modulated SMCs in low-ESS sites, resulting in collagen-poor plaques in regions exposed to proinflammatory ESS throughout their progression (Figure IX in the online-only Data Supplement).

Although lesions in atherosclerotic pigs very closely resemble clinical coronary atherosclerosis, extrapolation of our findings to humans still requires caution, given the severely hyperlipidemic and diabetic experimental conditions. The present findings resemble remarkably, however, the observations

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**Figure 6.** A. Relative mRNA levels of matrix-metalloproteinase (MMP)-1, MMP-13, MMP-14, tissue inhibitor of MMP (TIMP)-1, and the MMP/TIMP mRNA ratio in segments with persistently low endothelial shear stress (ESS) vs higher ESS. B. Immunostaining shows greater protein expression of MMP-1, MMP-8, and MMP-13 in representative segments with persistently low ESS (top) vs higher ESS (bottom). Asterisks denote the lumen. C. Quantification of MMP-1 immunostaining. D. Association of MMP-1 protein expression with the time-averaged ESS, best described by a negative logarithmic relationship.
from a large-scale clinical study (Prediction of Progression of Coronary Artery Disease and Clinical Outcome Using Vascular Profiling of Shear Stress and Wall Morphology) that demonstrated greater plaque enlargement in coronary regions with low ESS. Our current observations advance prior mechanistic studies directly linking low ESS to atherogenesis in vitro, and they complement previous clinical observations in several important ways: by profiling individual lesions serially in vivo throughout their evolution, we assessed for the first time the long-term effect of the temporally changing local ESS; we demonstrate how persistently low, eccentric ESS associates with the subsequent development of high-risk eTCA; and, most importantly, we explored in detail histopathologic features linking low ESS to the biology of high-risk plaque formation—an elusive goal in humans.

Our study might have benefited from a greater number of pigs. Its power increased, however, by profiling the entire length of 15 arteries at 5 consecutive time points and analyzing a total of 184 arterial segments. Although we may have introduced some selection bias in the samples we analyzed histologically, we were careful to include segments with various ESS trajectories over time, and selected about two thirds of all 304 computationally defined segments. Arteries were not perfusion-fixed under pressure to preserve the ability to

Figure 7. A, Upper row: CD45 and matrix-metalloproteinase (MMP)-1 immunostaining in serial sections of a representative low-endothelial shear stress (ESS) segment indicates marked MMP-1 expression, colocalization with inflammatory leukocytes, and intense green fluorescence by in situ zymography (ISZ) optimized for MMPs, indicating high collagenolytic activity. Picrosirius-red staining (right) shows reduced fibrillar collagen, and marked fibrous cap thinning (arrowheads), in the same segment. Lower row, Reduced leukocyte infiltration (left), absence of MMP-1 staining, attenuated MMP-mediated collagenolytic activity, and high collagen content (right) in serial sections of a representative higher ESS segment. Quantitative analyses of monocyte chemotactant protein (MCP)-1 mRNA levels (B), intimal leukocyte content (C), and percentage area of intimal fluorescence by ISZ optimized for MMP (D). E, Increased expression of MMP-8 (left), MMP-13 (middle), and MMP-14 (right) in serial sections of a representative low-ESS segment and colocalization with marked collagenolytic activity as indicated by ISZ (bottom; left). Addition of MMP-specific inhibitor EDTA abolishes zymographic activity (bottom; right). Quantification of the percentage of intimal area with fluorescence intensity, shown in red in the insets, affirms the 8-fold decrease of collagenolytic activity with the addition of EDTA in this lesion.
perform immunohistochemical and in situ zymographic analyses, which may have distorted the dimensions of the histological cross-sections.

In conclusion, this study provides important new insights into the in vivo role of low ESS in the evolution of early lesions to high-risk coronary plaques. Although local ESS may change substantially over time as plaques form and progress, eTCA, lesions particularly prone to rupture and trigger thrombotic events in humans, are highly unlikely to develop in coronary regions that are not exposed to persistently low, eccentric ESS throughout their long-term evolution. In these regions with persistently low ESS, the combination of attenuated collagen synthesis and enhanced MMP-mediated collagen breakdown favors reduced collagen content and substantial thinning of the fibrous cap—characteristics that compromise plaque stability.

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Disclosures

None.

References


Significance

Local hemodynamic conditions critically affect atherosclerotic plaque development. This natural history study advances the current appreciation of low endothelial shear stress as an important proatherogenic stimulus in several important ways. First, we demonstrate in a human-like, porcine model of coronary atherosclerosis that local endothelial shear stress may change substantially over time as plaques form and progress. Second, we show that persistently low, eccentric endothelial shear stress is a lesion-related factor conducive to subsequent development of eccentric thin-capped atheromata; high-risk plaques are highly unlikely to develop in the absence of these local characteristics. Third, we demonstrate that arterial regions exposed to persistently low endothelial shear stress develop plaques with reduced smooth muscle cell content, marked smooth muscle cell phenotypic modulation, attenuated collagen synthesis, and enhanced matrix-metalloproteinase–mediated collagen breakdown, thereby promoting the formation of collagen-poor, rupture-prone plaques. Early in vivo identification of high-risk lesions may guide the application of focused systemic treatments or selective local prophylactic interventions to avert the thrombotic complications of coronary plaque rupture.
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Materials and Methods

Study Design

The study protocol is summarized in Supplemental Figure I. The experimental protocol was approved by Harvard’s Institutional Animal Care and Use Committee, and conforms to the Guide for the Care and Use of Laboratory Animals. We analyzed animals used in a study investigating the natural history of coronary atherosclerosis in pigs.1 Five male, Yorkshire swine 12–14 weeks of age, initially weighing 18.9±1.0 kg, were sedated with Telazol (10 mg/kg) and injected via ear vein with filter-sterilized b-cell cytotoxin streptozotocin (50 mg/kg in 0.1 mol/l Na-citrate, pH 4.5) daily for 3 days to induce diabetes.1-4 Animals were subsequently given 25 g glucose twice daily at feeding for 2 days to offset insulin release from b-cells. Starting at the time of diabetes induction, the animals were fed a diet containing 1.5% cholesterol and 15% lard supplemented with sucrose, in quantities titrated to maintain serum total cholesterol (TC) and blood glucose (BG) levels between 500–800 mg/dl and 150–350 mg/dl, respectively, while allowing steady weight gain.4

Serial Cardiac Catheterizations

Coronary angiography and three-vessel intravascular ultrasound (IVUS) were serially performed in vivo in all major epicardial coronary arteries (left anterior descending; left circumflex; and right coronary artery) in all animals at weeks 4, 11, 16, 23, and 36.1 None of the animals died during the study period. Following final catheterization at week 36, the animals were euthanized, and the coronary arteries were harvested, frozen in liquid nitrogen, and stored at -80°C until further analysis by histopathology, immunohistochemistry, and RT-PCR.

Animals were fasted overnight before each catheterization procedure. Animals were at a surgical plane of anesthesia prior to each catheterization procedure, and remained under anesthesia for the entire procedure, as well as throughout euthanasia following the final procedure at week 36. The anesthesia protocol consisted of the combination of tiletamine with zolazepam (4.4 mg/kg, Telazol, Wyeth), Xylazine (2.2 mg/kg, Rompun, Bayer), and Atropine (0.05 mg/kg). After endotracheal intubation with 7-7.5mm sized tubes, the animals were ventilated with isoflurane (0.1% to 5.0%) and oxygen for maintenance of anesthesia throughout the procedure. Monitoring during each procedure included continuous electrocardiogram, arterial blood pressure and O2 saturation monitoring.

Access site for vascular access was the femoral groin area. The skin at the access site was aseptically prepared with povidone iodine (Betadine) solution. All incisions were made using a muscle-sparing technique and were kept as small as possible (~2.5 cm in length) in order to
minimize injury, facilitate fast healing and minimize post-operative pain. After the incision and the arteriotomy, an appropriate sized introducer sheath was placed in the artery. The sheath was removed immediately after completion of the procedure. All incisions were closed with a 3-layer closure of vicryl. Animals were monitored for post-operative pain and given analgesics (Buprenorphine, Buprenex, Reckitt & Colman, 0.05 mg/kg IM / BID for 24 hours, appropriately adjusted thereafter).

**Vascular Profiling for Endothelial Shear Stress (ESS) Calculation**

Intracoronary vascular profiling methodology has been previously described and validated *in vivo.* In brief, the 3D anatomy of each coronary artery was reconstructed from IVUS images and biplane coronary angiography. IVUS (ClearView, Boston Scientific, Natick, MA) was performed with automated pullback at 0.5 mm/sec. The arterial lumen and external elastic membrane (EEM) 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 EEM boundary points were connected by spline curves to rebuild the lumen and EEM 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 opacified blood to fill a known volume of coronary artery during a contrast injection. Blood was considered as Newtonian fluid, and its viscosity was estimated using the hematocrit and serum 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 with uniform inlet flow velocity. ESS at the lumen surface of the geometrically correct 3D reconstructed artery was calculated at all five time points as the product of viscosity and the gradient of blood velocity at the wall.

Each reconstructed artery was divided at week 36 into consecutive 3-mm-long segments along its length starting at the ostium, yielding a total of 304 computational segments from all 15 arteries. Although the plaques that developed at follow-up were often longer than 3mm, we chose 3-mm segments as our unit of measure, because this length was methodologically reliable and would accurately reflect the local hemodynamic and plaque characteristics, and the heterogeneous and highly focal changes occurring within the plaque over time. Readily visible side branches were identified on IVUS pullbacks and, subsequently, on the preserved arteries as reference markers to locate the same segments over time.
ESS was averaged in each 3-mm segment at all 5 time points, and was correlated to histopathology at follow-up. By dividing the arteries into short, 3-mm segments that show homogeneity of ESS\(^1\,^3\) and histopathologic characteristics,\(^1\) we eliminated the possible error of averaging. Although ESS is a continuous variable, for the present analyses we categorized ESS in individual segments at each time point as low ESS (<1.2 Pa) or higher ESS (intermediate/high; ≥1.2 Pa), based on previous experimental\(^1\,^2\) and human studies.\(^5\,^7\)

Because low ESS associates in a time- and level-dependent manner with the rate of subsequent plaque progression\(^1\,^5\,^7\,^12\) and with the magnitude of high-risk plaque characteristics,\(^2\,^3\) we identified segments that remained in persistently low ESS throughout their evolution, defined by time-averaged ESS <1.2 Pa through all five time points. All our analyses compared these segments by histology at follow-up to all other segments that developed in a higher ESS environment over time, defined by time-averaged ESS ≥1.2 Pa.

In addition to ESS magnitude we assessed circumferential ESS heterogeneity, which has also been linked to plaque composition and instability.\(^13\,^14\) We calculated in each segment and at each time-point an ESS eccentricity index according to the following equation:

\[
\text{ESS Eccentricity Index} = \frac{\text{ESSmax} - \text{ESSmin}}{\text{ESSmax}}
\]

where ESSmax represents the highest ESS, and ESSmin the lowest ESS around each segment’s circumference. To account for possible changes of ESS magnitude and circumferential distribution over time, we calculated a time-averaged ESS eccentricity index for all 5 time-points. Eccentric ESS was defined by an ESS eccentricity index ≥0.5.

**Assessment of Vascular Remodeling by IVUS**

The nature of the remodeling response to plaque growth was assessed by IVUS at follow-up in each 3mm-long segment. Remodeling was assessed in each segment by comparing the local remodeling behavior of each individual segment with the global remodeling response of the entire artery, as previously described.\(^1\,^3\,^15\) Briefly, the EEM areas of all the IVUS cross-sections along each reconstructed artery were plotted against the corresponding intima-media areas. The global reference of the entire reconstructed artery was determined by the linear regression line and its 90% prediction band in the EEM area vs. intima-media area plot. The EEM and intima-media area of each individual segment were then identified within the corresponding plot, and three local remodeling patterns were defined: (a) excessive expansive remodeling if the EEM area of the segment was above the upper limit of the 90% prediction band of the entire artery remodeling behavior, (b) compensatory expansive remodeling if the EEM area of the segment was within the 90% prediction band, and (c) constrictive remodeling if the EEM area of the
Histopathologic Analyses

We performed histopathologic analyses at follow-up in 184 segments that were carefully selected to represent different ESS trajectories over time and had a broad range of 3D geometry and plaque severity by IVUS. To ensure representative sampling, we chose 184 segments which had similar ESS distribution and similar plaque characteristics by IVUS over time as the 120 computational segments which were not analyzed by histology (Supplemental Figure II).

We cryosectioned the middle portion of each of the 184 segments at 7-μm thickness. We assessed the intima-to-media ratio (IMR) and lipid accumulation by Van Gieson elastin and Oil-Red-O staining, respectively. 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 10 regions along the length of the cap. We used the cap thickness at the thinnest region for our analyses. We analyzed fibrillar collagen content using picrosirius-red staining of sections viewed under polarized light.

Immunohistochemical Staining

Immunostaining for leukocytes used a monoclonal antibody against pig CD45 leukocyte common antigen (mouse anti-pig CD45, clone K252-1E4, AbD Serotec, Oxford, UK; 1:50). SMC content was assessed by immunostaining for SMC α-actin (HHF35, Enzo Life Sciences, Farmingdale, NY; 1:30). Immunostaining against MMP collagenases used primary antibodies against MMP-1 (R&D Systems; 1:20), MMP-8 (Clone 115-13D2, Millipore; 1:250), MMP-13 (Millipore; 1:30), and MMP-14 (Chemicon; 1:250). Secondary antibody staining was performed using the LSAB2 kit (DakoCytomation, Carpinteria, CA). AEC substrate (DakoCytomation) was used for detection of the HRP conjugate. Samples were counterstained in Mayer’s hematoxylin.

Quantitative analyses were performed on Image-Pro Plus 5.1 (Media Cybernetics, Bethesda, MD). The percentage of total intima area with positive color was calculated for each section. To account for differences in plaque size between lesions with different preceding ESS, we normalized all histological assays for plaque size by measuring the percent of the intima with positive staining.

Immunofluorescent Staining

Primary antibodies against MMP-1, MMP-8, MMP-13, and MMP-14 were applied to slides and incubated overnight at 4°C. Secondary antibodies labeled with AlexaFluor-488 or -594 (Invitrogen) were applied at 1:200 dilution for 2 hours. The samples were coverslipped with a DAPI-containing mounting
media (Vector Laboratories). The sections were imaged using an epifluorescent scope. Image processing and quantification were performed using Metamorph software (Molecular Devices, Inc.). Double immunofluorescent staining for the MMPs, and CD45 or SMC α-actin were performed to assess co-localization of these enzymes with their cellular sources — leukocytes or SMCs, respectively. Double immunofluorescent staining with α-actin (Enzo Life Sciences, 1:30) and desmin (Millipore, 1:30) was performed to assess the phenotype of intimal SMCs.

**Plaque Classification**

Categorization of segments into three plaque types used histomorphologic and histomorphometric criteria at follow-up, according to a modified classification of human plaques: (1) segments with minimal lesion, defined by IMR <0.2; (2) segments with intermediate lesion, defined by IMR ≥0.2 without evidence of a fibrous cap; and (3) segments with thin-capped atheroma (TCA) plaque morphology, defined by IMR ≥0.2 with evidence of a thin (<65 μm) cap overlying a large lipid core (>40% of the plaque size). Because an eccentric plaque pattern associates with plaque vulnerability and rupture in human pathology and in IVUS-based studies, we further classified TCA as eccentric (eTCA) or concentric TCA (cTCA). Maximal and minimal plaque thickness were measured in Van Gieson elastin-stained sections, and a plaque eccentricity index was calculated for each segments using the following equation:

$$\text{Plaque Eccentricity Index} = \frac{\text{maximal plaque thickness} - \text{mineral plaque thickness}}{\text{maximal plaque thickness}}$$

eTCA were defined by a plaque eccentricity index ≥0.5, and cTCA by an index <0.5.

Presence of a true necrotic core, and not merely of a lipid-rich pool, comprises a critical component of rupture-prone lesions. In all TCA we therefore distinguished necrotic cores, defined as regions that were positive by Oil-Red-O but negative by collagen staining, from lipid pools, defined as regions positive for both lipid and collagen staining.

**SMC Apoptosis**

SMC apoptosis was assessed by TUNEL staining and α-actin staining of adjacent sections of representative segments (n=28) with different preceding ESS. We used dUTP-digoxigenin incorporation (Millipore), detection with an alkaline phosphatase-conjugated antibody to digoxigenin, and development with 5-bromo-4-chloro-3-indolyl-phosphate/p-nitroblue tetrazolium (Vector). Negative controls (omission of TdT) and positive controls (mouse thymus sections) were included. Co-localization of apoptotic nuclei with SMCs was assessed by TUNEL staining and α-actin staining in serial sections. Results were
In addition, we assessed directly intimal SMC apoptosis by combining α-actin immunofluorescent staining with TUNEL staining analyzed by confocal microscopy. Frozen sections of low–ESS segments (n=4) fixed in 4% paraformaldehyde (PFA) were blocked in 4% normal horse serum + 2.5% BSA and incubated with mouse anti-alpha actin (HHF-35, 1:20, Enzo Life Sciences) overnight at +4°C, followed with anti-mouse Alexa 594 (1:500, Invitrogen). TUNEL staining for apoptosis was performed subsequently according to the manufacturer’s protocol (Roche cat# 11684817910). Sections were counterstained with Hoechst 33342 (Invitrogen, H21492) and coverslipped with Fluorescent Mounting Medium (DAKO, cat# S 3023). Staining was analyzed under the confocal microscope (Olympus FV1000, Tokyo, Japan).

In-Situ Zymography for MMP Collagenases

MMP-mediated collagenase activity was assessed and quantified in each section by in situ zymography (ISZ), which can visualize enzymatic activity of this family of enzymes by optimizing experimental conditions (pH and specific inhibitors). We added 20 µl of a mixture of DQ-Collagen (Invitrogen) in low-melting-temperature Agarose 1%, 50 Mm Tris pH 7.5, 300 Mm NaCl, 5 Mm CaCl2, 0.05% Brij-35, and 20 µM ZnCl2 on 10-µm-thick sections, coverslipped, and incubated the sections for 48 hours at room temperature. The contribution of metalloproteinases to collagenase activity was inhibited by the addition of the metalloenzyme inhibitor EDTA (20 mM). Sections were examined under a fluorescent microscope, with all images captured under the same settings and shutter conditions. The percentage of fluorescence intensity in each section was quantified (Image-Pro Plus 5.1 software), applying the same threshold for all images. All ISZ experiments were done in duplicate.

Transmission Electron Microscopic (TEM) Study

TEM analysis qualitatively assessed collagen fiber density (FEI Tecnai Spirit). Frozen segments were cryosectioned to create 40-µm thick sections, affixed to glass slides, and promptly fixed via immersion in a phosphate-buffered fixative solution comprised of 4% glutaraldehyde, 0.5% paraformaldehyde, and 1% lanthanum nitrate. Sections were stained with Coomassie Blue to facilitate section visibility before being dehydrated and embedded in a 1:1 mixture of Embed 812 / Spurr’s resin mixture (Electron Microscopy Sciences, Hatfield, PA). Following polymerization, the resin was separated from the glass and each cross-section was cut using a diamond saw into four equally sized quadrants. Each quadrant was affixed to the tips of blank resin blocks and further sectioned into 100-nm sections with an
ultramicrotome (Leica Ultracut UCT). These thin sections were collected, post-stained in 5% uranyl acetate in distilled water, and imaged using a transmission electron microscope (FEI Tecnai Spirit).

**Gene Expression by RT-PCR**

The mRNA was harvested from the intima and media of all segments (n=184). Cryosections for each of the arterial segments were harvested, the adventitia and surrounding heart tissue were dissected, and mRNA from the intima and media was isolated using a commercially available kit (Qiagen). Following reverse transcription (Invitrogen, Carlsbad, CA), the mRNA encoding procollagen-I, MMP-1, MMP-13, and MMP-14, and their endogenous inhibitors (i.e., tissue inhibitors of MMP [TIMP]-1, -2), monocyte chemoattractant protein (MCP)-1, platelet-derived growth factor (PDGF), and markers of SMC differentiation (α-actin, desmin, myosin heavy chain, SMM22A) were measured by real-time RT-PCR for each segment. The mRNA encoding procollagen-I, MMP-1, MMP-13, MMP-14, tissue inhibitor of MMPs (TIMP)-1, TIMP-2, monocyte chemoattractant protein (MCP)-1, platelet-derived growth factor (PDGF), and markers of SMC differentiation (α-actin, desmin, smoothelin, myosin heavy chain, SMM22A) were measured by real-time reverse-transcriptase polymerase chain reaction for each segment. Real-time RT-PCR was performed using a SYBR green master mix and an Applied Biosystems 7900HT Sequence Detection System. Cycle conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 94°C and 1 minute at 60°C. Target gene mRNA levels were normalized to the “housekeeping” (GAPDH) mRNA level in each extract. The oligonucleotide primers used are shown in Supplemental Table I.

**Statistical Analyses**

Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL) or STATA version 11.1. Continuous variables are summarized as mean ± standard error of the mean (SEM); categorical variables, as actual numbers and percentages. Correlation between two continuous variables was measured with the Pearson r correlation coefficient. For analyses with both categorical independent and dependent variables, random effects logistic regression was employed. For analyses with a continuous dependent and a categorical independent variable, random effects analysis of variance was used. As observations were not statistically independent, the animal was specified as a random effect to account for the clustering of arteries within animals. Findings were considered statistically significant at the 0.05 level.
References


Supplemental Table I. Primer sequences for genes analyzed by RT-PCR.

<table>
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<tr>
<th>mRNA</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
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<td>TTGAGCTCAGGGATGACCTT</td>
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<td>MMP-1</td>
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</tr>
<tr>
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<td>TGCTCTGTGCGGTACTATGC</td>
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<tr>
<td>SMM22A</td>
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<td>MCP-1</td>
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<td>PDGF</td>
<td>CAGGCTGAAGATCTCCAGA</td>
<td>TTCCTGCCTCTGAGTGGAT</td>
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</table>

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MCP: monocyte chemoattractant protein; MMP: matrix metalloproteinase; PDGF: platelet-derived growth factor; SMMHC: smooth muscle myosin heavy chain; TIMP: tissue inhibitor of matrix metalloproteinase.
Supplemental Figure I. Overview of the study protocol.
Supplemental Figure II. Plaque characteristics by IVUS and ESS distribution were similar in the 184 segments which were selected for histopathologic analyses and the 120 segments which were not analyzed by histology, thereby affirming representative sampling. Maximal intima-media thickness (maxIMT) by IVUS at follow-up (A); proportion of segments with significant plaque by IVUS at follow-up, defined as maxIMT≥0.5mm (B); proportion of segments with each grade of plaque severity by IVUS at follow-up (C); and plaque area by IVUS at follow-up (D) in the 184 segments which were selected for histological analyses vs. the 120 segments which were not analyzed by histology. Proportion of segments with low time-averaged ESS <1.2 Pa (E) and distribution across levels of time-averaged ESS (F) for the 184 segments which were selected for histological analyses vs. the 120 segments which were not analyzed by histology.
Supplemental Table I. Sensitivity, specificity, positive, and negative predictive value of persistently low ESS over time (weeks 4→36) in predicting eTCA plaque morphology at follow-up (week 36).

<table>
<thead>
<tr>
<th>Plaque type at follow-up</th>
<th>eTCA (n=31)</th>
<th>Non-eTCA (n=153)</th>
<th></th>
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<tbody>
<tr>
<td>Persistently Low ESS</td>
<td>23</td>
<td>52</td>
<td>Positive Predictive Value 23/75 = 30.7%</td>
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<tr>
<td>(n=75)</td>
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<tr>
<td>Higher ESS</td>
<td>8</td>
<td>101</td>
<td>Negative Predictive Value 101/109 = 92.7%</td>
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<tr>
<td>(n=109)</td>
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<td></td>
<td></td>
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<tr>
<td>Sensitivity</td>
<td>23/31 = 74.2%</td>
<td>Specificity 101/153 = 66%</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Figures

**Supplemental Figure I.** Local ESS in individual segments (n=184) frequently changed from low ESS (<1.2 Pa; lower row) to higher ESS (intermediate/high, ≥1.2 Pa; upper row) between consecutive time points. The respective arrows indicate the numbers and percentages of segments at each time point that evolved to each ESS category at the next time point. A substantial proportion of low-ESS segments, ranging between 12.4% and 47.3% over time, changed to higher ESS at the next time point. Similarly, a proportion ranging between 14% and 44.3% of segments with higher ESS over time changed to low ESS at the next time point. The black portion of the pie charts at each time point represents the proportion of segments with low ESS at the immediately preceding time point; the white portion represents the proportion of segments with higher ESS at the preceding time point.
Supplemental Figure II. Representative example of an arterial segment that developed in persistently low ESS. 

A, Two-dimensional maps show the ESS distribution along the artery length at weeks 4, 11, 16, 23, and 36. In each map, the horizontal axis represents the artery circumference (°) and the vertical axis represents the artery length (mm). The red rectangle at the most proximal portion of the artery includes an arterial segment which is exposed to persistently low ESS throughout its natural history, as indicated by the deep blue color in the ESS maps. B, Two-dimensional maps show the external elastic membrane (EEM) radius distribution over time along the same artery as above; in each map, the horizontal axis denotes the artery circumference (°) and the vertical axis the artery length (mm). The red rectangle includes the same proximal segment as in A. As plaque grows in this arterial segment, the EEM gradually increases in response to the growing plaque (as indicated by the change of color from blue to yellow to orange to ultimately red in the EEM maps). C, Evolution of the nature of arterial remodeling (red line) and of the local ESS over time (black line) in the same segment as above. The wall’s remodeling response changes from compensatory expansive to excessive expansive, which correlates with the increasing EEM dimensions and with the persistently low ESS (<1.2 Pa) at all time-points.
Supplemental Figure III. Representative example of an arterial segment that originated from low ESS but changed to higher ESS. A, Two-dimensional maps show the ESS distribution along the artery length at weeks 4, 11, 16, 23, and 36. In each map, the horizontal axis represents the artery circumference (°) and the vertical axis represents the artery length (mm). The red rectangle at the most proximal portion of the artery includes an arterial segment which is exposed to variable ESS throughout its natural history, as indicated by the different colors in the ESS maps at the different time-points. B, Two-dimensional maps show the external elastic membrane (EEM) radius distribution over time along the same artery as above; in each map, the horizontal axis denotes the artery circumference (°) and the vertical axis the artery length (mm). The red rectangle includes the same proximal segment as in A. As plaque grows in this arterial segment, the EEM initially decreases (blue color in the EEM map at week 11), which correlates with a substantial increase of the local ESS at the same time point (orange color in the ESS map). Further on, plaque progression is accompanied by gradual increase of the EEM (weeks 16 and 23) as indicated by the orange–red color in the corresponding maps and, consistently, with a relative reduction of the local ESS compared to week 11. Following week 23, the EEM again decreases (likely because the plaque growth surpasses the arterial wall’s capacity to expand further), and this segment ends up at week 36 with an EEM that is smaller than at baseline. Consistently, local ESS increases further at final week 36. C, Evolution of the nature of arterial remodeling (red line) and of the local ESS over time (black line) in the same segment as above. The wall’s remodeling response changes from compensatory expansive to constrictive, which correlates with the decreasing EEM dimensions and with the change of ESS from low to higher ESS.
Supplemental Figure IV. A, Evolution of external elastic membrane (EEM), lumen, and plaque area over time in all 184 segments. B, ESS over time in all 184 segments. Note the substantial increase of lumen and EEM area from week 11 to week 16, which correlates with the decrease of ESS from week 11 to week 16.
Supplemental Figure V. A, Proportion of (excessive or compensatory) expansive remodeling vs. constrictive remodeling in segments with TCA vs. non-TCA plaque morphology at follow-up. B, Proportion of TCA vs. non-TCA plaque morphology in segments with (excessive or compensatory) expansive vs. constrictive remodeling at follow-up.
**Supplemental Figure VI.** A, Compared to segments with higher ESS, segments with persistently low ESS over time had a greater proportion of excessive expansive remodeling and a lower proportion of constrictive remodeling by IVUS at follow-up. Lumen area (B) and plaque area (C) by IVUS at follow-up were greater in segments with persistently low vs. higher ESS over time.
**Supplemental Figure VII.** Histopathologic characteristics are homogeneous along the length of the 3mm-long arterial segments. Intimal area, lipid content, and leukocyte content were compared between pairs of sections that were obtained from the same segment, but from different portions (proximal vs. distal) of randomly selected segments (n=20). Note the striking similarity of Van Gieson elastin staining (A, B), Oil-red-O staining (D, E), and CD45 immunostaining outcomes (G, H) in the proximal vs. distal sections of representative segments, and the excellent correlation of the quantitative analyses of intimal area (C), lipid content (F) and leukocyte content (I) between all pairs of sections (n=20). These analyses affirm that histological findings measured in a single cross section of each 3mm-long segment are representative of the entire segment.
Supplemental Figure VIII. Double immunofluorescent staining co-localizes MMP-13 (red) (A) and MMP-14 (red) (B) to CD45-positive leukocytes (green) as indicated by the orange color in the merged panels (right), indicating that inflammatory cells comprise the main source of these enzymes. (C) MMP-8 (red) co-localizes to SMC α-actin (green), as indicated by the orange color in the merged panel (right). Asterisks denote the lumen.
Supplemental Figure IX. Schematic presentation of proposed mechanisms whereby low ESS may promote reduced plaque collagen content, and thus enhance plaque vulnerability. In regions with low ESS, increased SMC apoptosis favors reduced intimal SMC content, which likely outweighs the increased collagen-producing capacity of the modulated (“synthetic”) SMCs, overall resulting in reduced collagen synthesis. In addition, low ESS favors the expression and activity of potent collagen-degrading enzymes, thereby promoting collagenolysis.