Atheroprone Hemodynamics Regulate Fibronectin Deposition to Create Positive Feedback That Sustains Endothelial Inflammation

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Rationale: The extracellular matrix protein fibronectin (FN) is focally deposited in regions of atherosclerosis, where it contributes to inflammatory signaling.

Objective: To elucidate the mechanism by which FN deposition is regulated by local shear stress patterns, its dependence on platelet-endothelial cell adhesion molecule (PECAM)-1 mechanotransduction and the role this pathway plays in sustaining an atheroprone/proinflammatory phenotype.

Methods and Results: Human endothelial cells were exposed in vitro to atheroprone or atheroprotective shear stress patterns derived from human carotid arteries. Onset of atheroprotective flow induced a transient increase in FN deposition, whereas atheroprone flow caused a steady increase in FN expression and integrin activation over time, leading to a significant and sustained increase in FN deposition relative to atheroprotective conditions. Comparing FN staining in ApoE$^{-/-}$ and ApoE$^{-/-}$PECAM$^{-/-}$ mice showed that PECAM-1 was essential for FN accumulation in atheroprone regions of the aortic arch. In vitro, small interfering RNA against PECAM-1 blocked the induction of FN and the activation of nuclear factor (NF)-κB by atheroprone flow, which was rescued by the addition of exogenous FN. Additionally, blocking NF-κB activation attenuated the flow-induced FN expression. Small interfering RNA against FN significantly reduced NF-κB activity, which was rescued by the addition of exogenous FN.

Conclusions: These results indicate that FN gene expression and assembly into matrix fibrils is induced by atheroprone fluid shear stress. This effect is mediated at least in part by the transcription factor NF-κB. Additionally, because FN promotes activation of NF-κB, atheroprone shear stress creates a positive feedback to maintain inflammation. (*Circ Res*. 2010;106:1703-1711.)

Key Words: hemodynamics ■ atherosclerosis ■ fibronectin

Atherosclerosis is a focal inflammatory disease of the vasculature, marked by chronic activation of the endothelium.[1] In regions of disease, inflammatory signaling is continuously stimulated by the local environment to promote disease progression. It is known that atherogenic environments tend to develop in bulbous, bending, or bifurcating locations in the vasculature that generally have low and oscillatory shear stress patterns compared to atheroprotective regions where shear stress is generally unidirectional and high.[2] Indeed, atheroprone and atheroprotective in vitro shear stress induce distinct endothelial phenotypes, and shear stress is among the early endothelial activators that lead to focal atherogenesis.[3,4]

Atherogenic shear stress patterns activate the inflammatory transcription factor nuclear factor (NF)-κB, whose downstream targets include multiple cytokines (monocyte chemoattractant protein [MCP]1, interleukin [IL]-8) and adhesion molecules (vascular cell adhesion molecule [VCAM], E-selectin), which together mediate recruitment of leukocytes necessary for lesion formation.[4–6] NF-κB activation is mediated by a shear stress mechanosensor, platelet-endothelial cell adhesion molecule (PECAM)-1.[7,8] PECAM is an intercellular junction protein that forms a mechanosensory complex with VE-Cadherin and VEGF receptor 2. This complex initiates many early signaling events in response to shear stress, including activation of MAP kinases and integrins.[7] PECAM is necessary for the activation of NF-κB, lesion formation, and vascular remodeling in atherogenic regions in PECAM$^{-/-}$ mouse models of atherosclerosis.[8–11] Therefore, shear stress activates endothelial inflammation via mechanotransduction in response to local atheroprone shear stress.

Atheroprone regions are also marked by differences in the extracellular matrix. The endothelium normally resides on an extracellular matrix composed primarily of collagen IV and

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Non-standard Abbreviations and Acronyms	
ApoE	apolipoprotein E
EC	endothelial cell
FN	fibronectin
IL	interleukin
MCP	monocyte chemoattractant protein
MMP	matrix metalloproteinase
NF-κB	nuclear factor κB
PECAM	platelet-endothelial cell adhesion molecule
VCAM	vascular cell adhesion molecule

laminin, except when injured.[12] By contrast, fibronectin (FN) is preferentially deposited within human plaques[13] and beneath the endothelium in wild type mice before lesion formation.[14] FN-null mice die of severe defects during embryonic development; however, insight has been gained from mice whose FN isoform, extra domain A, was either knocked-out or lacked splicing regulation. Both defects are predicted to result in reduced FN expression or matrix assembly[15,16] and both result in reduced atherosclerotic plaque burden.[17–19] FN is a provisional matrix component important in migration and wound healing. It promotes cell growth and motility[20] and regulates the deposition and assembly of other matrix components such as collagen.[21] In agreement with its transitory function, FN has been found to be remodeled relatively quickly based on both degradation by matrix metalloproteinases (MMPs) and FN endocytosis/secretion in cell culture systems.[22,23] FN binding, primarily (but not exclusively) by integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$, can influence outside-in signaling within endothelial cells (ECs), including regulation of NF-κB among other pathways.[24,25] Importantly, Orr et al found that endothelial cells plated on FN, but not on collagen or laminin, activated NF-κB in response to onset of shear stress, though the consequences of sustained stimulation by shear stress was not investigated.[14] In agreement, Chiang et al recently found that blocking FN in vivo reduced adhesion molecule expression and leukocyte infiltration into the vessel wall.[26] FN therefore seems to enhance inflammatory signaling within atheroprone regions.

Collectively, atheroprone regions have heightened inflammation, which is regulated by PECAM mechanotransduction and enhanced by local FN deposition. The goal of the present study was to determine whether fluid shear stress mediates the deposition of FN beneath the endothelium at atheroprone regions of arteries. These data reveal a positive feedback mechanism that could play a prominent role in the chronic inflammation and hence plaque formation in these regions. Interrupting this loop may therefore provide a novel approach for the prevention, regression or stabilization of atherosclerosis.

Methods

Mouse Models

All mouse studies were conducted with the approval of the University of Virginia Animal Care and Use Committee (ACUC no. 3597), and in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. To study the role of PECAM in FN deposition, tissue sections from atheroprone aortic arches of both $ApoE^{-/-}$ (n=3) and $ApoE^{-/-}PECAM^{-/-}$ (n=3) double knockout (DKO) mice (C57BL6; previously described[8]) and were euthanized and tissue acquired as described previously[8]. Paraffin embedded sections of aortic arches were immuno-stained for fibronectin (rabbit polyclonal, Sigma-Aldrich) and counterstained with hematoxylin either before animals were placed on a Western diet (age: 8 weeks; Chow diet) or after 14 weeks on the Western diet (age: 22 weeks).[8]

Cell Culture

Each experimental replicate was performed using human umbilical vein endothelial cells (ECs) from different single donors at passage 2, as previously described.[27] Human ECs were plated in M199 growth media (Biowhittaker) supplemented with 10% FBS (Hyclone), 5 mg/mL endothelial cell growth supplement (ECGS; Biomedical Technologies), 10 mg/mL heparin (Sigma), 2 mmol/L L-glutamine (Gibco), and 100 U penicillin/streptomycin (Invitrogen) at 80 000 cells/cm^2. Before flow, cells were washed in DPBS and the medium was exchanged with reduced serum medium (2% FBS and 4% dextran by weight to increase the viscosity).

In Vitro Hemodynamic Flow Model and Reagents

To investigate the role of shear stress patterns on FN deposition in vitro, a cone and plate flow device imposed shear stress waveforms derived from the human internal carotid sinus (atheroprone) or the common carotid artery (atheroprotective) as defined previously by our laboratory.[3,27] The atheroprotective waveform is pulsatile and unidirectional with time-averaged shear of 14.5 dyne/cm^2, whereas the atheroprone waveform is pulsatile and reversing with a time average of 0.22 dyne/cm^2 as shown in Online Figure I (A). All flow experiments were 24 hours unless noted otherwise. Some experiments used medium supplemented with 10 μg/mL exogenous FN isolated from human plasma as previously described,[28] 8 mmol/L BAY 11-7082 (Calbiochem), pan-MMP inhibitor GM 1489 (Calbiochem), integrin activating antibody TS2/16 (10 mg/mL), or 5 ng/mL interleukin (IL)-1β (PeproTech).

Immunoprecipitation, Western Blotting, Real-Time RT-PCR, and Viral Infection

To measure PECAM phosphorylation, cells were collected in ice-cold lysis buffer containing phosphatase and protease inhibitors (Sigma) and were immunoprecipitated for PECAM (Santa Cruz Biotechnology). After immunoprecipitation, protein was analyzed with 4G10 phospho-tyrosine antibody (Upstate) and normalized to total anti-PECAM (Santa Cruz Biotechnology). On completion of the flow experiments, the cells were immediately collected directly into SDS-MAPK sample buffer (Cell Signaling). Western blotting was used to measure relative FN (BD, 1:2000), VCAM (R&D, 1:500) and PECAM (Santa Cruz Biotechnology, 1:500) protein and normalized to α-tubulin (1:5000, Sigma-Aldrich) to control for loading and normalized to a control static or shear stress condition from each experiment to account for differences per cell donor. Total RNA was extracted and reverse transcribed to cDNA RT-PCR analysis. Primer sequences for FN, VCAM, E-selectin, IL-8, MCP-1, and β2-microglobulin have been previously published elsewhere[29,30] (Online Table I). Endothelial cells were infected with 50 multiplicities of infection adenovirus containing NF-κB–luciferase reporter (Vector Labs) and, in pertinent experiments, additionally infected with 100 multiplicities of infection of the IκB dominant negative construct for 16 to 24 hours before shear stress.

Small Interfering RNA Knockdown of Fibronectin and PECAM

PECAM small interfering (si)RNA transfection followed protocol as previously described.[8] Fibronectin was knocked down by treating approximately 3 million ECs with 1nmole of FN1 siRNA (L-009853 to 00, Dharmacon) or control (D-001810, Dharmacon) and 60 μL of oligofectamine in 6 mL of OptiMEM-I media for 5 hours. Following

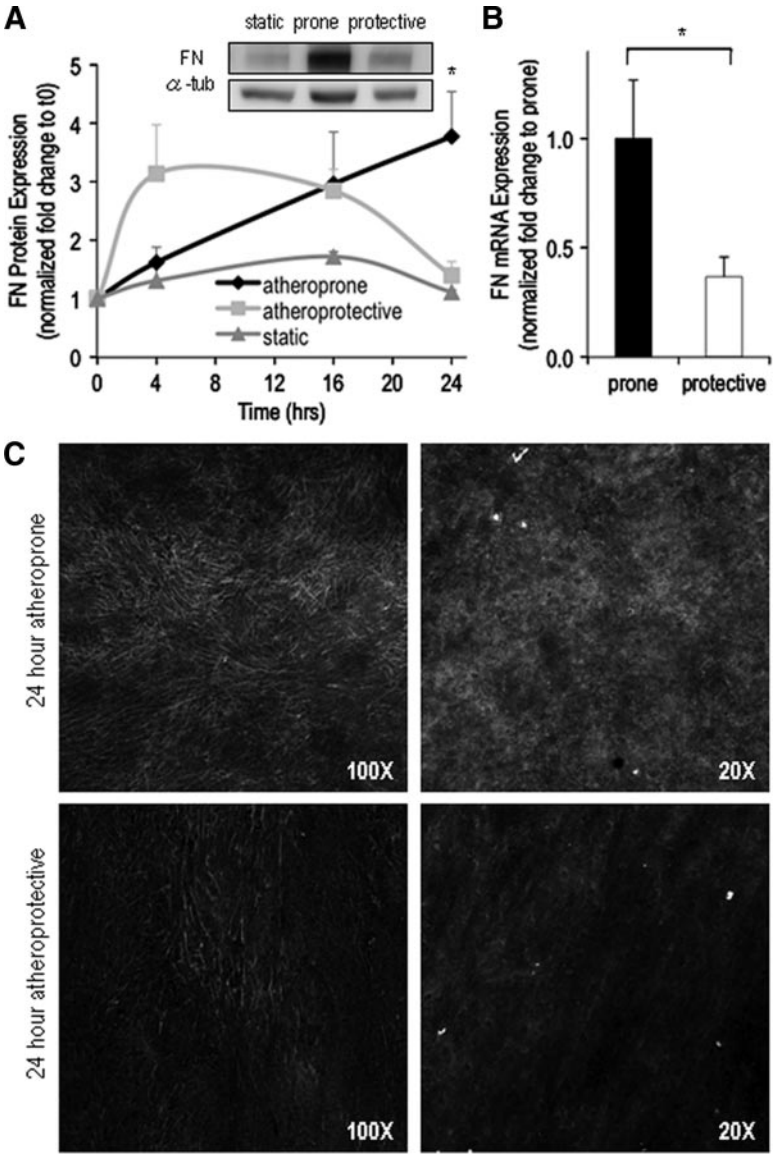

Figure 1. Physiological shear stress regulates FN deposition. A, ECs under atheroprone or atheroprotective shear stress or static conditions were analyzed for FN protein expression at 4, 16, and 24 hours and were normalized to α-tubulin and presented as fold changes relative to the initial condition (t0). A representative blot after 24 hours of flow is shown. *$P<0.05$ between flow types (2-way ANOVA). **B,** FN gene expression after 24 hours was assessed and presented as fold change relative to prone flow. *$P<0.05$ (Student's t test). **C,** FN staining after 24 hours at the indicated magnification following atheroprone or atheroprotective shear stress. Values are means±SE (n=5 to 10).

16 to 24 hours recovery post transfection, ECs were replated on 1% gelatin, infected as described above, and used after 24 hours.

Data Analysis and Statistics

For cases where the statistical comparison is between two populations of noncontrol samples, 2-way or 1-way ANOVA with Tukey post hoc test, or Student's t test was used. When comparisons were made to controls a one-sample t test was used to test the null hypothesis of the mean ratio equal to 1 (denoted as †). In all cases, data are presented as the means±SE, with significance at $P<0.05$.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Physiological Shear Stress Differentially Regulates Deposition of Fibronectin

Previous observations revealed that FN was preferentially deposited in the lesion-prone carotid bifurcation of the human and mouse vasculature.[13,14] Because of the focal nature of the FN deposition in vivo, we hypothesized that shear stress may regulate this local FN expression and matrix assembly by the endothelium. Human ECs were therefore exposed to either atheroprone or atheroprotective shear stress (Online Figure I, A) and FN expression was assessed. Atheroprotective shear stress caused an immediate increase in FN expression after four hours that returned to basal levels by 24 hours (Figure 1A). In contrast, atheroprone flow caused a steady increase in FN, resulting in significantly higher levels after 24 hours. This regulation is, in part, attributable to active production and secretion of FN by the endothelium, and correlated with higher levels of FN mRNA expression in atheroprone compared to atheroprotective flow at 24 hours (Figure 1B). Elevated deposition of FN by shear stress was not seen under steady flow conditions of the same time-average shear stress, indicating that shear stress magnitude alone does not govern this pathway (Online Figure I, B). Under both physiological flow conditions, ECs were capable of producing FN fibrils (Figure 1C). The fibrils under atheroprone flow, however, were mesh-like and dense without noticeable alignment. By comparison, FN fibrils under atheroprotective flow appeared sparser, but were aligned in the direction of flow.

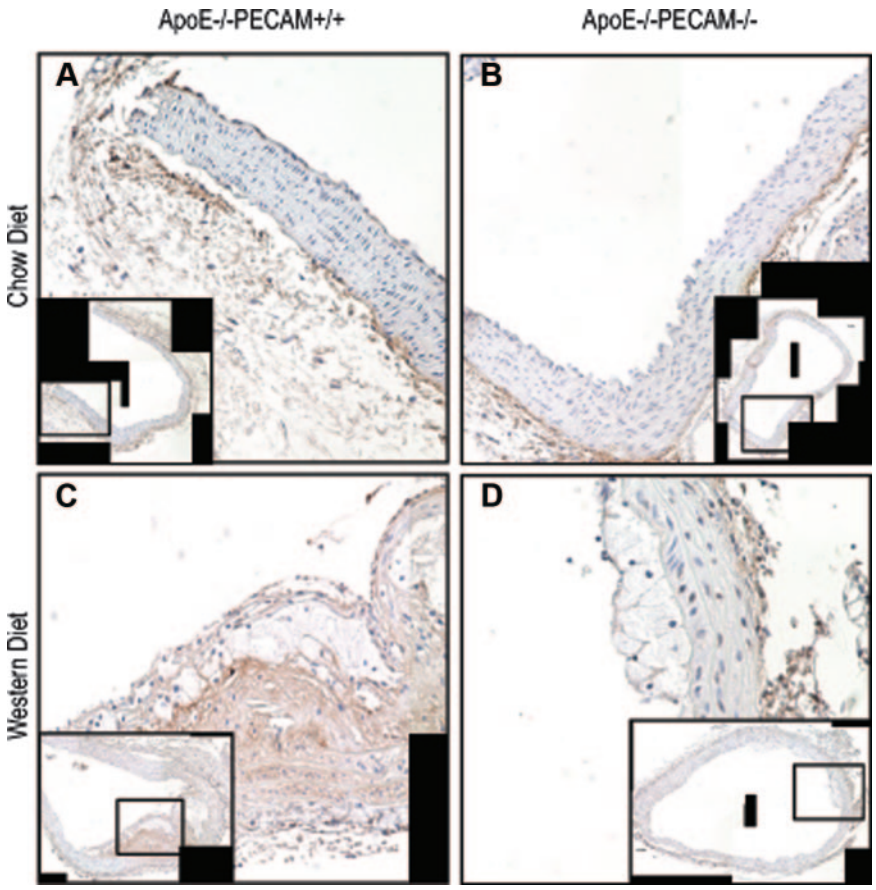

Figure 2. PECAM-1 promotes FN deposition in ApoE$^{-/-}$ mice. FN staining was compared between ApoE$^{-/-}$PECAM$^{+/+}$ and ApoE$^{-/-}$PECAM$^{-/-}$ mice in the aortic arch cross sections both before lesion formation (chow diet) and in advanced lesions (western diet) (representative images for 3 mice under each condition). Larger images display higher magnification of the full vessel cross-section showed in the inset.

PECAM Mediates Flow-Induced Fibronectin Deposition

Hypercholesterolemic mice lacking PECAM have reduced atherosclerosis, which correlates with reduced NF-κB activity.[8] Previous work also demonstrated that FN is deposited in atheroprone regions in wild type mice or young ApoE$^{-/-}$ mice preceding atherosclerosis development.[14] We hypothesize that the reduction in atherosclerosis in PECAM-deficient mice correlates with reduced FN expression at atheroprone regions. Tissue sections from the atheroprone aortic arch of both ApoE$^{-/-}$ and ApoE$^{-/-}$PECAM$^{-/-}$ double knockout (DKO) mice were stained for FN after fed either a chow diet or western diet (Figure 2). In ApoE$^{-/-}$PECAM$^{-/-}$ mice fed purely chow diet and before lesion formation, FN in the subendothelial matrix was absent compared to the ApoE$^{-/-}$. This result was more pronounced in ApoE$^{-/-}$ mice fed a western diet, which had large lesions showing strong staining for FN in the subendothelial matrix and within the atheroma, whereas in DKO mice, the lesions were smaller and those that did form were completely void of FN (Figure 2D). Therefore, regional FN deposition in atheroprone regions of mice preceding and during the progression of atherosclerosis is PECAM-dependent.

This result was corroborated in vitro using siRNA-mediated knockdown of PECAM that reduced PECAM expression to 30±9% of controls. Reduced PECAM was previously shown to block atheroprone flow-induced NF-κB activity and VCAM expression.[8] The knockdown of PECAM reduced FN protein deposition to 46±13% of controls and reduced NF-κB activity to 62±15% (Figure 3A through 3C). Adding exogenous FN to the cell culture medium of siPECAM cells restored FN incorporation into the subendothelial matrix and rescued NF-κB activation by atheroprone flow (Figure 3A through 3C). VCAM protein expression, a downstream target of NF-κB activity, was also restored by exogenous FN (Figure 3D). Therefore, PECAM-dependent induction of FN by flow is an important determinant of NF-κB activity in atheroprone environments. Interestingly, addition of exogenous FN to siControl samples did not significantly enhance NF-κB activity or VCAM expression, indicating that endogenously produced FN maximally supports this pathway.

To understand the mechanism by which PECAM differentially activates FN and NF-κB, the phosphorylation state of PECAM was assessed after 24 hours of atheroprotective, atheroprone or static conditions. Onset of shear stress has been reported to stimulate PECAM phosphorylation on tyrosines.[31] Figure 3E and 3F shows that atheroprone flow increased levels of PECAM phosphorylation after 24 hours, whereas in atheroprotective flow, phosphorylation was similar to static conditions. These results suggest that chronic atheroprone flow stimulates the PECAM pathway, thereby stimulating FN expression and inflammatory signaling.

Flow-Induced Fibronectin Deposition is NF-κB–Dependent

Fibronectin transcription was previously found to depend on NF-κB, which is able to bind and activate an NF-κB site in

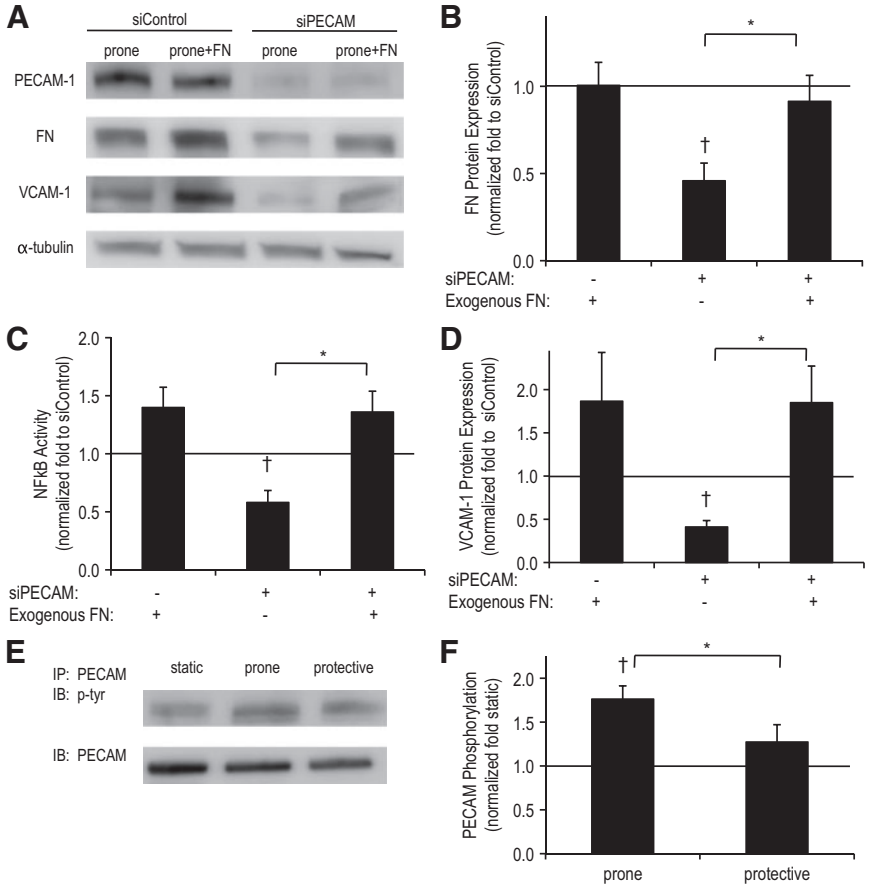

Figure 3. PECAM promotes atheroprone flow-induced NF-κB activity via FN deposition. A, PECAM (siPECAM)- or control siRNA (siControl)-treated ECs exposed to atheroprone flow were analyzed for protein expression by Western blot and normalized to α-tubulin. Each measurement was presented as fold change relative to the untreated siControl condition represented by the horizontal line at 1 **(B through D)**. FN protein levels (n=4 to 8) **(B)**, NF-κB luciferase reporter activity (n=4 to 7) **(C)**, and VCAM expression (n=4.6) **(D)** was assessed in ECs under atheroprone flow after siPECAM (+) or siControl (−), with or without exogenous FN. **E and F,** PECAM tyrosine phosphorylation following exposure to atheroprone or atheroprotective shear stress was measured (n=4) (F). Values are means±SE normalized to α-tubulin and presented as fold changes relative to the untreated siControl **(A through D)** or normalized total PECAM and presented as fold changes relative to the static condition **(E and F)**. †$P<0.05$ (1-sample t test), compared to untreated siControl **(B through D)** or static conditions **(F)**; *$P<0.05$ (1-way ANOVA).

the FN promoter.[32,33] We previously found that atheroprone flow activates NF-κB both in vivo and in vitro in a PECAM-dependent manner, similar to FN.[8] We therefore investigated whether NF-κB controls FN expression. We used both pharmacological inhibition (BAY 11-7082) and adenoviral delivery of dominant negative IκB to inhibit NF-κB. BAY (8 mmol/L) blocked NF-κB activity induced by atheroprone flow to 38±12% relative to the vehicle (DMSO), whereas dominant negative IκB reduced NF-κB activity to 14±4% relative to the empty-vector infection control (Figure 4). Despite not as efficiently reducing NF-κB, the BAY compound decreased FN protein deposition more affectively than the dominant negative IκB, suggesting that off-target effects may exist. However, both treatments confirmed that both FN mRNA and protein levels were NF-κB–dependent under atheroprone flow.

Reduced Integrin Activation Maintains Low Fibronectin Deposition Under Atheroprotective Flow

Figure 3 showed that depleting PECAM reduced FN expression and NF-κB activity in response to atheroprone flow, both which were rescued by exogenous FN. We therefore tested if ECs that expressed low levels of FN in atheroprotective flow could be induced to increase FN deposition and NF-κB activity. Following 16 hours of atheroprotective flow preconditioning, exogenous FN was added for the remaining 8 hours. Despite increased FN in the circulating media, FN assembly into matrix remained at low, basal levels similar to controls, and NF-κB was unchanged (Figure 5A and 5B). IL-1β-treated ECs resulted in a temporal activation of NF-κB relative to untreated controls (4 to 8 hours: 63.9±2.3-fold; 24 hours: 35.5±3.6-fold). Despite this level of activation, IL-1β treatment was not sufficient to induce FN deposition, similar to previous reports[34] (Figure 5C). This result suggests that factors other than low NF-κB activity under atheroprotective flow contribute to the low FN matrix assembly. Next, we tested whether MMPs may be involved in FN degradation.

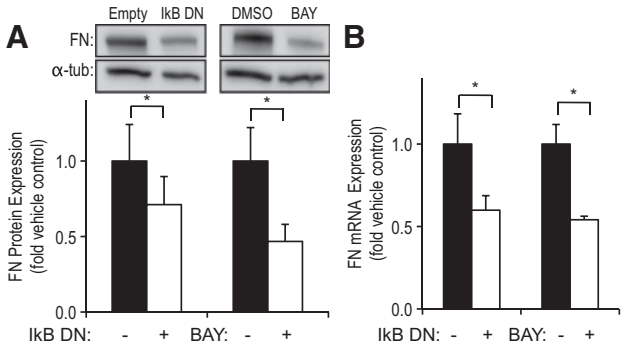

Figure 4. Atheroprone-induced FN deposition is NF-κB–dependent. NF-κB activity was blocked during 24 hours of atheroprone flow using either BAY pharmacological agent or with an IκB dominant negative construct, and FN protein **(A)** and gene expression **(B)** were assessed compared to the vehicle control. Values are means±SE (n=4) and reported as fold changes relative to the atheroprone control. *$P<0.05$ (paired t test).

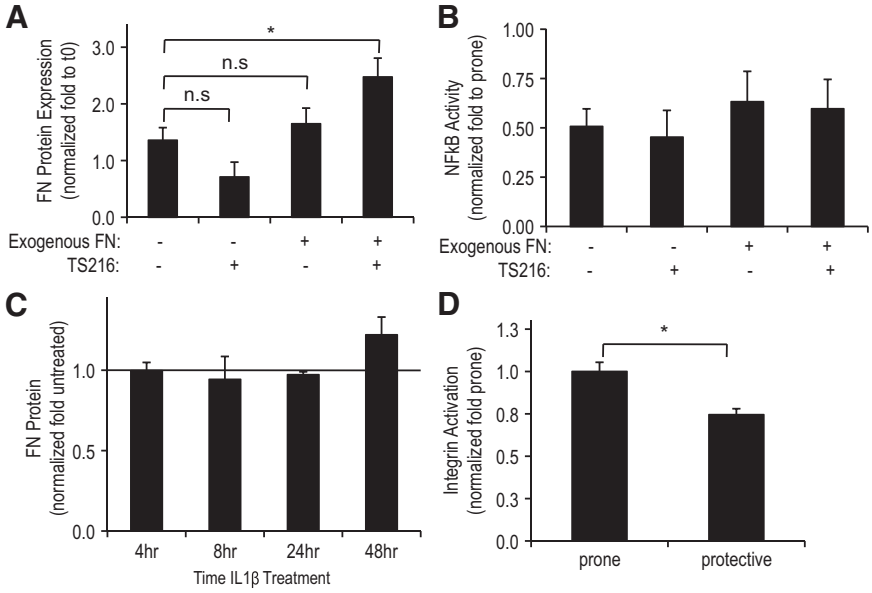

Figure 5. Atheroprotective flow maintains low, basal FN via reduced integrin activation. A and B, FN levels and NF-κB activity was assessed after 24 hours of atheroprotective flow with or without the addition of exogenous FN and/or integrin activating antibody TS2/16 for the last 8 hours of flow, and data were presented as fold change relative to the initial condition (t0) or to atheroprone control. **C,** ECs were treated with 5 ng/mL of IL-1β and FN was normalized to α-tubulin and compared to an untreated condition (represented by the horizontal line at 1). **D,** After 24 hours of shear stress, EC monolayers were incubated with 20 μg/mL GST-FNIII$_{9-11}$ for 30 minutes to measure integrin activity/binding and presented as fold change relative to the atheroprone condition. Values are means±SE (n=3 to 5). *$P<0.05$ compared to untreated control (**A and B**, 2-way ANOVA; **C**, 1-sample *t* test; **D**, Student's *t* test).

However, the temporal pattern of FN accumulation under atheroprotective flow was unaffected by the pan-MMP inhibitor GM1489 (Calbiochem), in agreement with other studies,[21] suggesting that this process is MMP-independent (Online Figure II). This result suggests that atheroprotective flow regulates matrix composition by mechanisms other than FN protein production or MMP-dependent degradation. Integrin activation is necessary for FN assembly,[35] and onset of laminar shear is known to transiently activate integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$.[14,36] We therefore considered whether atheroprone flow affects FN matrix assembly by controlling integrin activation. Integrin activity, assayed by binding FNIII$_{9-11}$ protein that is specific for high-affinity integrins, was elevated in atheroprone compared to atheroprotective flow (Figure 5D). To test the role of integrin activation in FN deposition, the integrin activating antibody, TS2/16,[37] was added simultaneously with exogenous FN. TS2/16 significantly increased FN deposition in cells under atheroprotective flow when added together with FN, but neither agent was active individually (Figure 5A). Significantly, despite increased levels of FN with the treatment of FN and TS2/16, NF-κB activity was unchanged (Figure 5B).

Fibronectin Enhances NF-κB Activity Under Atheroprone Flow

Previous reports showed that in ECs plated on FN, onset of laminar flow activated NF-κB, whereas cells on collagen did not; they also showed that sustained atheroprone shear stress activated NF-κB relative to atheroprotective.[8,14] We therefore assessed the contribution of FN to differential regulation of NF-κB by atheroprone and atheroprotective flow. First, to minimize cell-derived FN under atheroprone flow, siRNA was used to knockdown FN production in culture. Treatment with siRNA against FN (siFN) reduced prone-induced FN to 15±3% compared to control siRNA (siControl) (Figure 6A). In cultures treated with siFN, NF-κB reporter activity was significantly reduced by 22% after 24 hours of atheroprone flow (Figure 6B). Addition of exogenous FN (10 μg/mL) to the medium in both siControl and siFN treated cells resulted in a complete rescue in FN deposition (135±41%) and NF-κB activity to control levels. NF-κB–dependent gene transcription was also investigated between siFN and siControl conditions under atheroprone flow. Figure 6C shows that E-selectin, MCP-1, and IL-8, but not VCAM, were each significantly reduced compared to siControl.

Discussion

In vivo, deposition of FN beneath the endothelium correlates with activation of inflammatory pathways at disease-susceptible regions. In vitro, FN promotes activation of multiple inflammatory signaling pathways in endothelial cells.[14,38,39] Here we show that atheroprone shear stress can potently regulate FN expression and inflammation, providing strong evidence that focal FN deposition in vivo is attributable to regional hemodynamics. According to these and previous results, activation of inflammatory pathways in endothelial cells within atherogenic environments involves two independent events. First, the subendothelial matrix must be remodeled to replace the antiinflammatory basement membrane proteins such as collagen and laminin with a proinflammatory protein such as FN. Second, sustained signaling through integrins must be induced by flow patterns that prevent adaptation and downregulation of the inflammatory signals. The present data demonstrate that an atheroprotective flow inhibits both pathways, whereas atheroprone flow pattern can induce both arms of this response, and thus, is sufficient to induce sustained inflammatory activation of the endothelium.

Endothelial PECAM is a component of a mechanosensory complex that is necessary for activation of integrins and hence integrin-dependent pathways such as NF-κB under flow.[7,8,11] Previously, our group and others reported that in atherogenic mouse models (ApoE$^{-/-}$, LDLR$^{-/-}$, carotid ligation), deletion of PECAM reduced plaque burden in atheroprone regions, in association with decreased nuclear NF-κB.[8–11] Reducing PECAM in ECs in vitro also attenuated

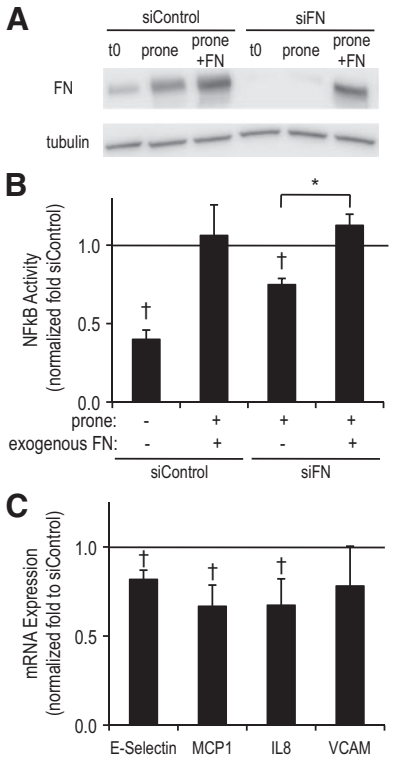

Figure 6. Increased FN deposition under atheroprone flow promotes NF-κB activity. A, ECs were treated with siRNA against FN (siFN), with or without addition of exogenous FN under static and atheroprone conditions and compared to untreated siRNA control (siControl). **B,** NF-κB reporter activity was compared between the siFN and siControl ECs after 24 hours of atheroprone flow and presented as fold change relative to the siControl untreated atheroprone condition (represented by the horizontal line at 1). **C,** Cells treated with siFN or siControl were exposed to atheroprone flow for 24 hours and then analyzed for expression NF-κB–dependent genes presented as fold changes relative to siControl (**horizontal line** at 1). Values are means±SE (n=6). †$P<0.05$ (1-sample t test), compared to the untreated siControl atheroprone condition, set to 1; *$P<0.05$ (1-way ANOVA).

NF-κB activation by atheroprone flow.[8,10] We now show that assembly of a FN matrix in vitro requires PECAM and this effect is mediated by NF-κB. Analysis of PECAM$^{-/-}$ mice also showed less FN in atheroprone regions of the aorta and reduced NF-κB. Although it is difficult to determine cause and effect in a mouse model, these results support the relevance of the in vitro observations. Thus, PECAM promotes induction of both FN expression and NF-κB activity in response to atheroprone flow to promote a sustained proinflammatory response.

Interestingly, Chen et al observed that flow-induced tissue remodeling, a process that is highly dependent on extracellular matrix and active in atherosclerosis, was reduced in PECAM$^{-/-}$ animals.[11] Blocking FN polymerization in the same carotid ligation model also similarly inhibited vessel remodeling and inflammation.[26] Thus, reduced FN may contribute to the remodeling defect in PECAM$^{-/-}$ mice, possibly by impairing the deposition of collagen or the inflammatory response, both of which are important components of many vascular remodeling processes.[21,26] The partial reductions seen after deleting or inhibiting PECAM or FN indicate that atherosclerosis can occur through independent pathways, however, they also strongly support these events as significant contributors.

Phosphorylation of PECAM on onset of laminar flow is believed to be a critical early event in mechanotransduction.[31] Our results now demonstrate that PECAM phosphorylation is sustained under atheroprone flow. Thus, PECAM phosphorylation may be relevant to in vivo-like, sustained phenotypes.[3,8,29,40] Regions that develop atherosclerosis are subject to shear stress patterns that involve flow reversal, which may continuously activate PECAM and its downstream targets. By contrast, unidirectional flow (characteristic of atheroprotective regions) may allow PECAM pathways to adapt or desensitize at later times. Therefore, differential PECAM activation may explain the observation in mice and in vitro, that PECAM had significant effects on lesion formation and cell signaling in some hemodynamic environments, but not others.[8–10,41]

An important implication of our data are that the NF-κB–dependent increase in FN under atheroprone flow, together with the ability of FN to promote NF-κB activation, creates a positive-feedback circuit. This circuit would be predicted to enhance both NF-κB activation and FN deposition, thereby producing sustained, progressive inflammation. One would predict the existence of such positive feedback mechanisms in lifelong, progressive inflammatory conditions such as atherosclerosis. The importance of FN in this disease is also supported by results from genetically modified mice where FN splicing was altered.[18,19]

In our siRNA experiments, FN-depleted cells incorporated exogenous FN into the extracellular matrix. By contrast, adding exogenous FN to control cells had little effect. This finding is particularly noteworthy for cells under atheroprotective flow, which at 24 hours showed low levels of FN in the matrix but no increase on addition of FN to the medium (Figure 5A). This result implies that FN gene expression is only one factor determining FN matrix assembly; cells under atheroprotective flow must fail to assemble the FN into fibrils for other reasons. This idea is particularly interesting in light of the fact that FN concentration in the plasma is high, yet FN deposition into the matrix occurs mainly at atheroprone regions in vivo.

In this regard, it is known that only conformationally activated, high-affinity integrins mediate FN matrix assembly.[35] We therefore examined integrin activation in atheroprone versus atheroprotective flow. Indeed, integrin activity was significantly higher in cells under atheroprone flow, which may mediate the elevated FN deposition under this condition. Furthermore, rescuing reduced FN deposition under atheroprotective flow synergistically required addition of exogenous FN and activation of integrins, supporting the necessity for both arms of the pathway, as noted above. Surprisingly, even when FN deposition was increased under atheroprotective flow, albeit not to atheroprone-induced levels, NF-κB activity remained low. This result suggests that FN alone is not sufficient to activate inflammatory signaling, but that it enhances flow-dependent signaling. These findings provide evidence that the regional hemodynamic environ-

ment dictates local subendothelial FN matrix assembly by regulating both transcription and fibril assembly.

Here we show that the increased FN in atherosclerosis-susceptible regions could enhance inflammatory signaling in the endothelium. Furthermore, substantial evidence suggests that fibronectin could favor inflammatory signaling throughout the vessel wall. Binding of monocytes to FN causes induction of the potent inflammatory cytokine IL-1β.[42,43] Exposure of the endothelium to cytokines (such as IL-1β) can further activate local inflammation as the endothelium expresses adhesion molecules to attract and recruit other inflammatory cells.[1] The dramatic difference in FN in the medial layer between WT and PECAM$^{-/-}$ vessels on Western diet (Figure 2C and 2D) suggests that endothelial mechanotransduction might also indirectly affect FN deposition and phenotypic modulation in the surrounding smooth muscle. FN also regulates vascular wall thickening, by increasing cellular (likely smooth muscle) proliferation and infiltration within the vessel wall: a hallmark of both early remodeling and advanced plaques.[26] Therefore, increased FN beneath the endothelium could have further deleterious effects within the vessel wall, subsequent to endothelial activation.

It has long been appreciated that the hemodynamic environment is a critical determinant of endothelial pro- or antiinflammatory phenotype in atherosclerosis. Here, we begin to elucidate the role that shear stress mechanotransduction plays in matrix remodeling. Our results show that increased FN deposition is an important event during induction of an inflammatory phenotype by atheroprone flow. PECAM is the key mechanosensor in this pathway, and NF-κB forms a positive feedback circuit that establishes an inflammatory state that can progress over time. Further investigation of how different local environmental cues (mechanical forces, extracellular matrix, soluble factors) interact will be crucial for understanding of how inflammatory signaling becomes chronic and promotes the progression of atherosclerosis.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Fibronectin (FN), a component of the extracellular matrix, localizes to atherosclerotic lesions in humans and contributes to atherosclerosis lesion formation in atherogenic mouse models.
- Even before lesion formation, FN is preferentially deposited in regions of injury, but is it not present in healthy regions of the vasculature. Mechanisms underlying the regional regulation of focal deposition remain unclear and their contribution to atherogenesis has not been assessed.

What New Information Does This Article Contribute?

- This study shows that deposition of FN and activation of inflammatory mediators within different locations of the vasculature is tightly governed by "mechanosensing" of the local hemodynamic shear stress patterns by the endothelium.
- Shear stress from regions of disease causes the expression and deposition of FN via PECAM-1 (platelet-endothelial cell adhesion molecule 1) signaling and the inflammatory transcription factor nuclear factor κB. Conversely FN in these regions promotes inflammation, including nuclear factor κB activation, thus establishing a proinflammatory positive-feedback loop that sustains inflammation in atherosclerotic lesions.

Atherosclerosis is a progressive inflammatory disease that develops within specific locations of the vasculature. Hemodynamic changes in diseased regions stimulate inflammatory signaling that predispose these regions to the development of atherosclerotic lesions. This study has revealed one way in which the endothelium remodels the extracellular matrix that further enhances the local flow-induced inflammatory signaling. This signaling cascade creates a "sustaining-feedback" effect that exacerbates the local inflammatory state of the endothelium. Breaking this inflammatory cycle may be a therapeutic strategy for slowing the progression of atherosclerosis.

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Atheroprone Hemodynamics Regulate Fibronectin Deposition to Create Positive Feedback That Sustains Endothelial Inflammation

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Supplemental Methods:

Mouse Models

All mouse studies were conducted with the approval of the University of Virginia Animal Care and Use Committee (ACUC#3597), and in accordance with the NIH recommendation outlined in "Guide for the Care and Use of Laboratory Animals". To study the role of PECAM in FN deposition tissue sections from atheroprone aortic arches of both ApoE-/- (n=3) and ApoE-/- PECAM-/- (n=3) double knockout (DKO) mice (C57BL6; *previously described*[1]) and were euthanized and tissue acquired as described previously[1]. Paraffin embedded sections of aortic arches were immuno-stained for fibronectin (rabbit polyclonal, Sigma-Aldrich) and counterstained with hematoxylin either before animals were placed on a Western diet (age: 8 wk, Chow diet) or after 14 weeks on the Western diet (age: 22 wk)[1].

Cell Culture

Each experimental replicate was performed using human umbilical vein endothelial cells (ECs) from different single donors at passage 2, as previously described[2]. Research from our group and others support the use of umbilical vein ECs in atherosclerosis-based flow models [1, 3-6]. Human ECs were plated in M199 growth media (Biowhitaker) supplemented with 10% fetal bovine serum (FBS; Hyclone), 5mg/ml endothelial cell growth supplement (ECGS; Biomedical Technologies), 10mg/ml heparin (Sigma), 2mM L-glutamine (Gibco), and 100U penicillin/streptomycin (Invitrogen) at 80,000 cells/cm^2 on surface treated plastic coated with 1% gelatin and allowed to grow to confluence over 18-24 hours. Prior to flow, cells were washed in DPBS and the medium was exchanged with reduced serum medium (M199 supplemented with 2% FBS, 5ug/ml ECGS, 10ug/ml heparin, 2mM L-glutamine, 100U penicillin/streptomycin, and 4% dextran by weight to increase the viscosity).

In Vitro Hemodynamic Flow Model & Reagents

To investigate the role of shear stress patterns on FN deposition *in vitro*, a cone and plate flow device imposed shear stress waveforms derived from the human internal carotid sinus (atheroprone) or the common carotid artery (atheroprotective) as defined previously by our lab[2, 4]. The atheroprotective waveform is pulsatile and unidirectional with time-averaged shear of 14.5dyne/cm^2, while the atheroprone waveform is pulsatile and reversing with a time average of 0.22dyne/cm^2 as shown in **Online Figure IA**. The flow device provides exchange of fresh medium at times greater than 4 hours, as well as a continuously controlled environment to maintain humidity, gas, and temperature at 37°C[2]. All flow experiments were 24 hours unless

noted otherwise. Some experiments used medium supplemented with exogenous FN isolated from human plasma as previously described[7], 8mM BAY 11-7082 (Calbiochem), pan-MMP inhibitor GM 1489 (Calbiochem), integrin activating antibody TS2/16 (10mg/ml), or 5ng/ml IL-1β (PeproTech). To measure integrin activity, EC monolayers were incubated with 20 μg/ml GST-$FNIII_{9-11}$ for 30 minutes after shear stress[8]. Cells were then washed, lysed and immunoblotted using anti-GST antibody (SantaCruz) and normalized to α-tubulin (Sigma-Aldrich). For FN staining, cells were immediately fixed using 4% paraformaldhyde and permeablized using 0.2% Triton X. Cells were stained using FN antibody (BD, 1:200) at room temperature for 1 hour, followed by 1 hour incubation with goat anti-mouse secondary conjugated to fluorophore (Invitrogen, 1:300). Slides were imaged using Nikon Eclipse C1 confocal microscope.

Immunoprecipitation and Western Blotting

To measure PECAM phosphorylation, cells were collected in ice-cold lysis buffer containing phosphatase and protease inhibitors (Sigma). Samples were immunoprecipitated (IP) with 20ml of anti-PECAM (SantaCruz) and 50ml of Dynabead- Protein G according to the manufacturer's protocol (Invitrogen). After IP, protein was analyzed with 4G10 phospho-tyrosine antibody (Upstate) and normalized to total anti-PECAM (SantaCruz) to control for loading and results were presented normalized to the static condition.

Upon completion of the flow experiments, the cells were immediately collected directly into SDS-MAPK sample buffer (Cell Signaling). Western blotting was used to measure relative FN (BD, 1:2000), VCAM (R&D, 1:500) and PECAM (Santa Cruz, 1:500) protein and normalized to α-tubulin (1:5000, Sigma-Aldrich) to control for loading and normalized to a control static or shear stress condition from each experiment to account for differences per cell donor.

RNA Isolation and Real-Time RT-PCR Analysis

Total RNA was extracted using PureLink RNA Purification System (Invitrogen) as described by the manufacturer, and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad). Primer sequences for FN, VCAM, E-Selectin, IL-8, MCP-1, and β2-microglobulin (B2M) have been previously published elsewhere [9, 10] (Supplemental Table 1). The expression of mRNA was analyzed via real-time reverse transcriptase polymerase chain reaction (RT-PCR) using SYBR master mix (Roche Diagnostics) and an iCycler (Bio-Rad). Gene cycle numbers were normalized to B2M and presented as fold results to flow conditions as specified.

Viral Infection of NF-κB reporter and IκB dominant-negative

Endothelial cells were infected with 50 MOI adenovirus containing NF-κB-luciferase reporter (Vector Labs) and, in pertinent experiments, additionally infected with 100 MOI of the IκB dominant negative construct for 16-24 hours prior to the onset of shear stress. At the conclusion of experiments, cells were lysed in cold passive lysis buffer (Promega) and frozen at -80C until luciferase assay was performed.

siRNA Knockdown of Fibronectin and PECAM

For depleting PECAM, ECs were treated with 330pmol of control (D-001810, Dharmacon) or human PECAM siRNA (L-017029-00, Dharmacon) and 20μL oligofectamine (Invitrogen) in 3mL of OptiMEM-I medium for 5 hours. Cells were then transferred to fresh medium and incubated for 48 hours prior to flow as previously described[1]. Fibronectin was knocked down by treating approximately 3 million ECs with 1nmole of FN1 siRNA (L-009853-00, Dharmacon) or control (D-001810, Dharmacon) and 60ml of oligofectamine in 6ml of OptiMEM-I media for 5 hours. Following 16-24 hours recovery post transfection, ECs were replated on 1% gelatin, infected as described above, and used after 24 hours.

Data Analysis and Statistics

Cells from different primary donors were used for each experiment, which adds inherent variance to the data. To account for this "batch effect" all raw data was normalized to loading controls as specified above (α-tubulin for protein, β2M for mRNA, total protein for reporter assays) followed by normalizing to a donor-specific static or flow control for each experiment, as noted. For cases where the statistical comparison is between two populations of non-control samples, two-way or one-way ANOVA with Tukey post-hoc test, or student's t-test was utilized. When comparisons were made to controls a one-sample t-test was used to test the null hypothesis of the mean ratio equal to 1 (denoted †). In all cases, data is presented as the mean±standard error, with significance at $p<0.05$.

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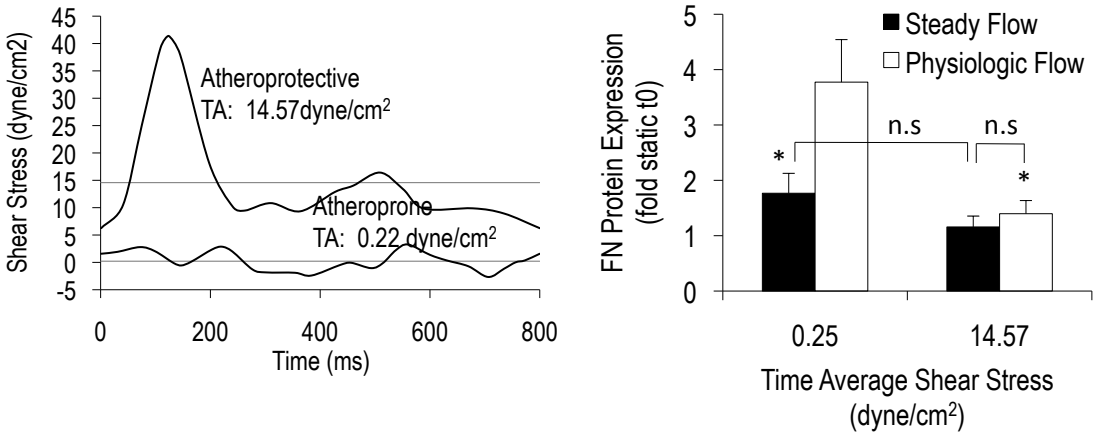
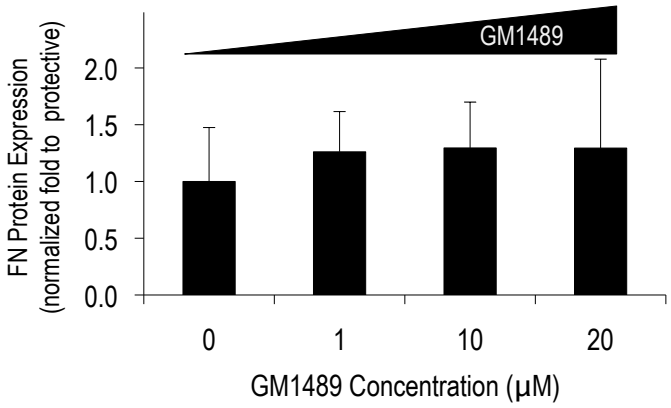


Online Figure I. Human Atheroprone and Atheroprotective Shear Stress Waveforms Regulate FN Deposition. A. One cardiac cycle is shown for each shear stress waveform that was derived from the internal carotid sinus (atheroprone) and common carotid artery (atheroprotective) along with its time-average (TA) shear stress. B. FN deposition was compared after 24 hours of steady or physiologic (atheroprone or atheroprotective) shear stress at low (0.22 dyne/cm^2) and high (14.57 dyne/cm^2) time-average shear stress. Statistics: Values are means±SE,*=$p<0.05$ two-way ANOVA compared to 0.22 dyne/cm^2 physiologic flow (atheroprone).



Online Figure II. Fibronectin degradation under Atheroprotective Flow is MMP-independent. ECs were treated with GM1489 pan-MMP inhibitor at the point of peak FN expression (4hrs) under atheroprotective flow for the remaining 20 hours and FN deposition was assessed, and presented normalized and fold to protective condition.

Gene	Sense Primer	Anti-sense Primer	Temp (C°)
β2-Microglobulin	AGCATTCGGGCCGAGATGTCT	CTGCTGGATGACGTGAGTAAACCT	60-63
E-Selectin	AATCCCAGTTTGTGAAGCTTTCCA	GCCAGAAGCACTAGGAAGACAATT	63
Fibronectin	AACGATCAGGACACAAGGAC	CCTCTCACACTTCCACTCTC	61
IL8	CATGACTTCCAAGCTGGCCG	TTTATGAATTCTCAGCCCTC	63
MCP1	CCAGCAGCAAGTGTCCCAAAG	TGCTTGTCCAGGTGGTCCATG	63
VCAM1	GTTTGTCAGGCTAAGTTACATATTGATGA	GGGCAACATTGACATAAAGTGTTT	60

Table 1. Primer Sequences. Forward and reverse primer sequences for analyzed genes and optimal temperature.