Shear stress modulates VCAM-1 expression in response to TNF-α and dietary lipids via interferon regulatory factor-1 in cultured endothelium

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DeVerse JS, Sandhu AS, Mendoza N, Edwards CM, Sun C, Simon SI, Passerini AG. Shear stress modulates VCAM-1 expression in response to TNF-α and dietary lipids via interferon regulatory factor-1 (IRF)-1 in cultured endothelium. Am J Physiol Heart Circ Physiol 305: H1149–H1157, 2013. First published August 9, 2013; doi:10.1152/ajpheart.00311.2013.—Dyslipidemia is a primary risk factor for cardiovascular disease, but the specific mechanisms that determine the localization of atherosclerotic plaques in arteries are not well defined. Triglyceride-rich lipoproteins (TGRL) isolated from human plasma after a high-fat meal modulate TNF-α-induced VCAM-1 expression in cultured human aortic endothelial cells (HAECs) via an interferon regulatory factor (IRF)-1-dependent transcriptional mechanism. We examined whether fluid shear stress acts as a mediator of IRF-1-dependent VCAM-1 expression in response to cytokine and dietary lipids. IRF-1 and VCAM-1 were examined by immunofluorescence in TNF-α-stimulated HAEC monolayers exposed to TGRL and a linear gradient of shear stress ranging from 0 to 16 dyn/cm² in a microfluidic device. Shear stress alone modulated TNF-α-induced VCAM-1 expression, eliciting a 150% increase at low shear stress (2 dyn/cm²) and a 70% decrease at high shear stress (12 dyn/cm²) relative to static. These differences correlated with a 60% increase in IRF-1 expression under low shear stress and a 40% decrease under high shear stress. The addition of TGRL along with cytokine activated a fourfold increase in VCAM-1 expression and a twofold increase in IRF-1 expression. The combined effect of shear stress and TGRL on the upregulation of membrane VCAM-1 was abolished by transfection of HAECs with IRF-1-specific small interfering RNA. In a healthy swine model, elevated levels of endothelial IRF-1 were also observed within atherosusceptible regions of the aorta by Western blot analysis and immunohistochemistry, implicating arterial hemodynamics in the regulation of IRF-1 expression. These data demonstrate direct roles for fluid shear stress and postprandial TGRL from human serum in the regulation of IRF-1 expression and downstream inflammatory responses in HAECs.

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atherosclerosis is a multifactorial disease characterized by the focal accumulation of lipid-rich plaques at arterial sites that correlate strongly with regions of disturbed blood flow. Diets high in saturated and nonesterified fats contribute to a state of metabolic dysregulation that is associated with chronic low-grade inflammation and activation of stress responses that accelerate the progression of atherosclerosis (18, 21). Elevated levels of circulating lipoproteins and cytokines, such as TNF-α, contribute to inflammatory responses that culminate in the preferential recruitment of leukocytes to the endothelium via the upregulation of cell adhesion molecules, including VCAM-1 (24). Despite the recognition that dyslipidemia is a condition that predisposes an individual to cardiovascular disease, the specific mechanisms that drive diet-induced inflammation associated with endothelial upregulation of VCAM-1 and the initiation of atherosclerotic plaques are not well defined.

Hypertriglyceridemia is strongly correlated with the current trend in the pervasiveness of obesity, insulin resistance, type II diabetes, and metabolic syndrome, which places more than one-third of Americans at enhanced risk for cardiovascular disease (26). We (36, 44) have recently reported that triglyceride-rich lipoproteins (TGRL) isolated from obese and hypertriglyceridemic subjects after a high-fat meal were proatherogenic to human aortic endothelial cells (HAECs) in that they increased VCAM-1-dependent monocyte adhesion relative to TNF-α stimulation (36, 44). In contrast, TGRL from subjects with normal serum triglycerides did not upregulate inflammation. In fact, some subjects’ TGRL actually exerted an antiatherogenic effect in that they suppressed VCAM-1 expression and monocyte adhesion relative to TNF-α stimulation. The changes in VCAM-1 expression elicited by TGRL were dependent on the expression and activity of the transcription factor interferon regulatory factor (IRF)-1.

IRF-1 regulates key genes involved in immune responses, cell growth, and inflammation (35, 38). Constitutively expressed in quiescent ECs, IRF-1 is upregulated by TNF-α and acts cooperatively with NF-κB to regulate VCAM-1 expression in response to cytokine stimulation (30). IRF-1 expression increases in oxidized LDL-treated THP-1 macrophages and correlates with oxidized LDL content in the plaques of high-cholesterol diet-fed pigs (20). It was also found to play a role in the induction of IL-8 by 25-hydroxycholesterol in the cultured human endothelium and in a mouse model of atherosclerosis (43). Taken together, this evidence suggests that IRF-1 is regulated by lipid uptake and metabolism and may play a role in diet-induced atherosclerosis. However, it is unknown precisely how IRF-1 activity and the resultant VCAM-1 expression by the endothelium are regulated by diet in the context of other factors that locally modulate inflammation.

Arterial hemodynamics are known to exert distinct signals that directly affect the endothelial inflammatory phenotype (31). The local hydrodynamic forces acting on the endothelium, such as the shear stress induced by blood flow, strongly correlate with the distribution of atherosclerosis in arteries (16). Arterial geometries such as branch points, bifurcations, and curvatures are more susceptible to atherosclerosis. The endothelium at those sites is subjected to disturbed laminar flow profiles, characterized by low-magnitude time-average wall shear stress (≤5 dyn/cm²), large temporal and spatial
shear stress gradients, and flow reversal. Conversely, straight unbranching arteries are considered atheroresistant and are characterized by high-magnitude time-average shear stress (≥10 dyn/cm²), low shear stress gradients, and unidirectional pulsatile flow. A causal link between disturbed flow and atherosclerosis has been demonstrated by studies in atherosclerotic mouse models subjected to surgical manipulation of the carotid artery through partial ligation (28) or perivascular casting (5). These studies have demonstrated that low-magnitude and oscillatory shear stress are essential for plaque formation. However, while shear stress has been established as a determinant of the endothelial inflammatory phenotype, its influence alone is not sufficient to induce pathological changes in the endothelium. Rather, it is believed to contribute to an unsteady state of equilibrium in endothelial function, which can be tipped toward atherosclerosis in the presence of risk factors that contribute to systemic inflammation (12).

VCAM-1 expression is highly correlated with the predisposition to and development of atherosclerosis. Previous studies (24, 25) have established the importance of VCAM-1 as an early marker of inflammation that is localized to atherosclerotic lesions and plays a critical role in the recruitment of monocytes to the endothelium. The functional significance of VCAM-1 expression in precipitating atherosclerosis has been demonstrated by a reduced atherosclerotic burden in high-cholesterol diet-fed apolipoprotein (Apo)E-deficient mice as a consequence of blockade of monocyte binding to VCAM-1 (22). It has been well documented that shear stress modulates TNF-α-induced VCAM-1 expression in ECs, but the underlying mechanisms are not fully understood. Moreover, key questions remain as to how shear stress converges with diet-related factors to regulate EC function and inflammation. Motivating the present study is the finding that postprandial TGRL modulates VCAM-1 expression in an IRF-1-dependent manner (36), highlighting a potential role for IRF-1 in diet-induced inflammation and the susceptibility to atherosclerosis. We hypothesized that fluid shear stress and TGRL can each exert a distinct influence on cytokine-induced inflammation in the endothelium by directly modulating IRF-1 expression. Here, we used primary cultured HAECs exposed to a continuous spatial gradient in shear stress to assess the relative importance of hydrodynamics and a subject’s postprandial TGRL as regulators of VCAM-1 expression via IRF-1.

MATERIALS AND METHODS

**TGRl isolation and characterization.** Human subjects were recruited according to Institutional Review Board-approved protocols at the University of California-Davis. TGRl were collected and characterized as previously described (36, 44). Briefly, venous blood was collected after a 12-h overnight fast and again 3.5 h after a standardized moderately high-fat meal (1,230 calories, 42% from fat and 32% from saturated fat). TGRl (p < 1.0063 g/ml) were isolated by ultracentrifugation and normalized for ApoB content as quantified by ELISA.

**Cell culture and treatment protocol.** HAECs (Genlantis) were maintained in endothelial growth medium-2 (Lonza) with 10% FBS and 1× antibiotic-antimycotic solution (Invitrogen) and used for experiments at passages 5–7. To stimulate inflammation, HAECs were treated with TNF-α (0.3 ng/ml, R&D Systems) alone or in combination with TGRl (10 mg/dl ApoB) for 4 h (VCAM-1), 2 h (IRF-1), or 1 h (NF-κB). The dose of TNF-α represents the EC50 for VCAM-1 expression as measured by flow cytometry (44). Treatments without TGRl were supplemented with an equal amount of buffer in which the isolated TGRl was suspended (196 mM NaCl and 0.3 mM EDTA) to compensate for any changes in volume and media composition.

**Flow cytometry.** TGRl were characterized for their atherogenic potential by assaying VCAM-1 surface expression relative to TNF-α in static culture. HAECs were detached using enzyme-free cell dissociation buffer (GIBCO), labeled with a fluorescein-conjugated antibody to human VCAM-1 (14 μg/ml, BBA22, R&D Systems), and analyzed by FACSscan flow cytometer (Becton Dickinson).

**Shear flow experiments.** To assess a wide range of shear stress magnitudes while constrained by limited quantities of TGRl from a given subject, hydrodynamic experiments were performed on HAEC monolayers using a microfluidic flow chamber based on Hele-Shaw stagnation flow theory (41) as previously described (13, 40). The geometry of the channel (see Fig. 1A) generates a linear decrease in shear stress magnitude along the centerline of the longitudinal axis for a given flow rate. The channel width is a function of the axial distance (x) and increases toward the flow exit. The wall shear stress (τw) in the channel can be described as follows:

\[ \tau_w = \frac{\mu Q}{w_1 R (1 - \frac{x}{L})} \]

where \( \mu \) is the viscosity of the flow medium, Q is the flow rate, h is the channel height, w1 is the width of the inlet, and L is the length of the channel. HAECs were seeded on collagen type I-coated (100 μg/ml, Invitrogen) glass coverslips and grown to 80–90% confluency. PDMS microfluidic chambers were then sealed to the HAEC monolayers via a vacuum network. Monolayers were exposed to flow driven by a Masterflex L/S peristaltic pump (Cole-Parmer) in a humidified chamber heated to 37°C. Leibovitz-15 medium (GIBCO), supplemented with 10% FBS, endothelial BulletKit (Lonza), and 1× antibiotic-antimycotic solution, was used as the flow medium to maintain pH in the absence of CO2. Leibovitz-15 media had no deleterious effect on cell viability or TNF-α-induced VCAM-1 expression in HAECs. The addition of TGRl at 10 mg/dl ApoB did not significantly affect the viscosity of the flow media (~0.8 cP at 37°C).

**Immunofluorescence staining.** Sheared cells were rinsed with PBS and fixed in 1% paraformaldehyde (Electron Microscopy Sciences) for VCAM-1 analysis or 4% paraformaldehyde and 0.3% Triton X-100 (Sigma-Aldrich) for IRF-1 and NF-κB. Samples were blocked in 10% donkey serum (Invitrogen) and 1% human serum albumin (ZLB Behring) and incubated with monoclonal anti-VCAM-1 (no. 3987, Cell Signaling). Samples were then incubated with secondary antibody-conjugated anti-mouse IgG and anti-rabbit IgG (7.5 μg/ml, no. 111-505-003, Jackson ImmunoResearch). Coverslips were mounted using VECTASHIELD mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories). Images from three representative fields taken at ×100 magnification were averaged at each shear stress magnitude, corresponding to fixed axial position x. Mean fluorescence intensity was quantified using ImageJ software (National Institutes of Health).

**Cell transfection.** Cells (1 × 10⁶) were transfected with 150 pmol small interfering (si)RNA targeting human IRF-1 (sc-35706, Santa Cruz Biotechnology) or scrambled siRNA as a control (sc-37007, Santa Cruz Biotechnology) using a 4D-Nucleofector system (Lonza). After 48 h, cells were treated as described above.

**Porcine endothelial sample collection and immunohistochemistry.** Fresh arterial samples from healthy adult swine were obtained from a local abattoir, and ECs were isolated from a ~1-cm² region located at the inner curve and lateral walls of the aortic arch (a representative atherosusceptible site) and separately from the dorsal descending thoracic aorta (a representative atheroresistant site) as previously described (32). Samples were transfected directly to protein lysis.
buffer. Additional tissue samples were fixed in 10% formalin, paraffin embedded, and sectioned for immunohistochemistry. Samples were rehydrated, blocked for endogenous peroxidase, and heated at 98°C to an active state. Following rehydration, samples were incubated with biotinylated goat anti-rabbit (0.14 μg/ml, sc-497, Santa Cruz Biotechnology). Samples were subsequently incubated with biotinyl-rabbit polyclonal anti-IRF-1 antibody (2 μg/ml, sc-497, Jackson Immunoresearch), incubated with SuperSignal West Pico and Femto Chemiluminescent Substrate (1:4 ratio, Pierce), and developed using the ChemiDoc MP gel imaging system (Bio-Rad). Bands were quantified by densitometry using Image Lab software (Bio-Rad).

Data analysis. Data were analyzed using GraphPad Prism (version 5.0) software. In general, multiple treatment groups were compared by ANOVA, and differences were assessed by a Student-Newman-Keuls posttest. Two experimental groups were compared using Student’s t-test (pairing where appropriate). Two-tailed P values of < 0.05 were considered statistically significant unless otherwise indicated.

RESULTS

Shear stress superposed with TGRL modulates TNF-α-induced VCAM-1 expression. To dissect the relative contributions of dietary lipids and hydrodynamic shear stress to the regulation of IRF-1-induced inflammation, we used cultured primary aortic ECs exposed to a spatially varying flow field. We used a microfluidic device that was vacuum sealed directly to an endothelial monolayer. The sidewalls of the chamber narrow in a systematic way to vary the local wall shear stress in a linear manner with the axial position along the direction of fluid flow (40). The flow rate was chosen to deliver a gradient in shear stress that spanned a physiological range from 16 dyn/cm² at the inlet down to virtually 0 dyn/cm² at the stagnation (static) point just proximal to the outlet of the channel. VCAM-1 expression on HAECs conditioned with TNF-α in the presence and absence of TGRL was detected by immunofluorescence and quantified at intervals along the shear gradient corresponding to 0, 2, 4, 8, and 12 dyn/cm² within the same monolayer (Fig. 1A). Shear stress alone did not affect basal VCAM-1 expression in unstimulated HAECs, which did not register significantly above the detection limit measured by labeling with an isotype IgG control antibody. TNF-α significantly increased VCAM-1 expression, which varied spatially as a function of shear stress magnitude over the length of the channel. The peak in expression at 2 dyn/cm² marked the most dramatic enhancement of VCAM-1 expression with respect to the static condition near the channel outlet. VCAM-1 remained significantly elevated at 4 dyn/cm², but receptor number continuously decreased with position down the channel to a level equivalent to the static condition at 8 dyn/cm². Near the channel inlet corresponding to a level of 12 dyn/cm², there was a 70% reduction in VCAM-1 relative to the outlet at 0 dyn/cm². To determine whether the response to steady laminar shear stress was dominated by a transient response to the acute onset of flow, we assessed the impact of a longer duration of shear stress exposure by conditioning HAECs over 24 h in the Hele-Shaw device before stimulating samples with TNF-α during the last 4 h. The pattern in VCAM-1 expression in response to TNF-α was consistent with that observed over 4 h of shear in that peak expression was observed at 2 dyn/cm² and...
suppression was detected at 12 dyn/cm² (Fig. 1B). Thus, ECs exhibit the capacity to alter their inflammatory response as a function of small increments in the magnitude of shear stress, increasing VCAM-1 expression by approximately twofold over distances of ~2 mm.

We next examined the capacity for TGRL, isolated from plasma after consumption of a high-fat meal, to modulate the cytokine-induced inflammatory response to shear. TGRL were categorized as proatherogenic (pro-TGRL) or antiatherogenic (anti-TGRL) defined by their capacity to up- or downregulate VCAM-1 surface expression, respectively, relative to TNF-α stimulation under static culture (36). We (36, 44) have previously documented that TGRL did not elicit an increase in VCAM-1 from HAECs in the absence of TNF-α stimulation regardless of its atherogenic activity in its presence. Similarly, we found that TGRL alone did not mediate activation with shear alone, motivating our focus on the superposition of TGRL and cytokine. In TNF-α-stimulated cells exposed to flow, pro-TGRL effectively doubled VCAM-1 expression relative to TNF-α alone while maintaining the same pattern of response over the range in shear stress (Fig. 2). VCAM-1 expression reached a maximum value approximately fourfold above stimulation with pro-TGRL and TNF-α under static conditions. This amounted to a ~120% increase in VCAM-1 over TNF-α alone at 2 dyn/cm², which persisted at 12 dyn/cm², where a larger approximately threefold increase was detected that was also significantly elevated relative to the static condition (Fig. 2). In contrast, superposition of anti-TGRL with TNF-α actually diminished VCAM-1 expression by ~50% over the range in shear stress. This effect was significant at 2 and 4 dyn/cm², but not at higher values of shear stress, which exerted a predominant inhibitory influence on VCAM-1 up-regulation, reducing it to levels below the static condition. Maximum suppression in VCAM-1 upregulation in the presence of anti-TGRL coincided with the peak in expression at 2 dyn/cm²; however, this inhibition did not significantly decrease VCAM-1 below that stimulated by TNF-α alone. Overall, these data demonstrate that both shear stress and TGRL have modulatory effects on cytokine-induced VCAM-1 expression.

Notably, TGRL from hypertriglyceridemic subjects enhanced the inflammatory response of HAECs to TNF-α under low shear stress conditions that promote VCAM-1 expression as well as offset the protective influence of high-magnitude shear stress that effectively suppresses inflammatory VCAM-1 up-regulation.

Shear stress is a regulator of IRF-1 expression. IRF-1 expression accounts for the relative atherogenicity of TGRL by modulating inflammatory upregulation of VCAM-1 during transcription (36). This motivated us to examine the extent to which shear stress itself modulated changes in transcriptional regulation. Quantifying the immunofluorescence of nuclear IRF-1 along the centerline of the channel revealed that shear stress did not significantly alter its basal expression in the absence of cytokine stimulation. However, nuclear IRF-1 expression increased significantly at shear stress ≤ 4 dyn/cm² in the presence of TNF-α stimulation (Fig. 3A). This spatial pattern of elevated IRF-1 expression mirrored that of VCAM-1 in that its rise was highest at 2 dyn/cm² (i.e., 60% above static) and steadily decreased up the channel, such that above 8 dyn/cm², expression was suppressed 40% below that of TNF-α under the static condition.

Consistent with the observed pattern in VCAM-1 expression along the channel, costimulation with pro-TGRL and TNF-α significantly increased the overall expression of nuclear IRF-1 (Fig. 3B). The maximum expression of nuclear IRF-1 (i.e., 2-fold above TNF-α static) coincided with VCAM-1 membrane expression of HAECs exposed to 2 dyn/cm². Moreover, pro-TGRL predominated over the inhibitory effect of high shear stress by increasing IRF-1 expression at 12 dyn/cm², where its expression was comparable with inflammation under the static condition. An important distinction of the influence of pro-TGRL on nuclear IRF-1 compared with VCAM-1 expression was that exposure to pro-TGRL alone in the absence of cytokine induced a modest but significant increase in nuclear IRF-1 at shear stress below 4 dyn/cm². Together, these data demonstrate that pro-TGRL and shear stress exert distinct effects that superease to augment expression of VCAM-1 along the Hele-Shaw channel.

Cytokine induces NF-κB activation independent of shear stress. IRF-1 is known to act cooperatively with NF-κB in dynamic transcriptional regulation of VCAM-1 expression after cytokine stimulation (30). We (36) have previously demonstrated that the ability of TGRL to modulate VCAM-1 expression was dependent on the relative level of IRF-1 activity but independent of changes in NF-κB activation. To assess the relative importance of shear stress-mediated changes in NF-κB versus IRF-1 on TNF-α-stimulated VCAM-1 expression, we examined nuclear translocation of the NF-κB p65 subunit by immunofluorescence. After 1 h of exposure to TNF-α and shear in the flow channel, p65 exhibited a predominantly nuclear localization compared with untreated HAECs, where it remained primarily cytoplasmic. In contrast to IRF-1, the level of p65 nuclear translocation was indistinguishable across the range of shear stress from 0 to 12 dyn/cm² (Fig. 3C) and did not correlate with the pattern of VCAM-1 expression. These data confirm that nuclear NF-κB is associated with cytokine induction of VCAM-1 but indicate that shear stress effects on its nuclear translocation did not factor prominently into the observed modulation of VCAM-1 along the channel.
Shear stress and TGRL each modulate VCAM-1 via an IRF-1-dependent mechanism. To demonstrate a direct role for IRF-1 in the regulation of cytokine-induced inflammation by shear, we examined VCAM-1 expression while suppressing IRF-1 using siRNA. Transfection with siRNA targeting human IRF-1 resulted in a marked reduction in VCAM-1 across the shear gradient, consistent with the cooperative role of IRF-1 in the cytokine-induced response (Fig. 4A). In the presence of a scrambled control siRNA, TNF-α induced VCAM-1 expression was similar to that shown in Fig. 1 and likewise enhanced at shear stress \( \leq 4 \text{ dyn/cm}^2 \) relative to the static region. However, the peak in VCAM-1 expression observed at 2 dyn/cm\(^2\) was abolished with siRNA targeting human IRF-1, demonstrating a direct role for shear stress in modulating the cytokine response via IRF-1. In the presence of siRNA targeting human IRF-1, costimulation with pro-TGRL failed to enhance VCAM-1 expression above that of TNF-α alone (Fig. 4B). Together, these data demonstrate that TGRL and shear stress converge specifically through IRF-1 to modulate VCAM-1, combining to promote maximum expression at a low shear stress.

**IRF-1 is differentially expressed in the porcine aortic endothelium.** To determine whether IRF-1 is also spatially regulated by arterial hemodynamics and may be a precipitating factor in the subsequent VCAM-1 upregulation, we examined its expression in a swine model free of atherosclerosis (Fig. 5A). We sampled endothelium from the inner curvature of the aortic arch, which is associated with disturbed flow characteristics, rendering this region of artery atherosusceptible, and compared this with a straight segment in the descending thoracic aorta associated with relatively undisturbed flow and atheroresistant activity (32). Endothelial IRF-1 expression by Western blot analysis was 50% greater in the atherosusceptible region (Fig. 5B). Enhanced endothelial expression was also observed by immunohistochemistry (Fig. 5, C and D). This pattern of differential expression is consistent with our observation of elevated IRF-1 expression and function at low shear stress in vitro and further implicates arterial hemodynamics in its regulation. Together our results suggest a putative role for IRF-1 as a mediator of inflammation that contributes to focal atherosusceptibility.

**DISCUSSION**

Atherosclerosis is a disease of complex etiology influenced by diet and an individual’s genetic makeup, which under pathological conditions can lead to metabolic dysregulation and the upregulation of circulating inflammatory mediators (24). A strong correlation exists between arterial hemodynamics and the localization of atherosclerotic lesions within the arterial tree. A wealth of published data has revealed a consistent pattern showing that the endothelium responds focally to low and oscillatory shear stress by promoting VCAM-1 expression and plaque progression in arterial regions of curvature.

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**Fig. 3.** Shear stress mediates interferon regulatory factor (IRF)-1 expression but not NF-κB translocation. A: representative images and quantification of IRF-1 in HAECs exposed to a linearly varying shear field in the presence and absence of TNF-α for 2 h. Values are means ± SE; \( n = 5–6 \). *P < 0.003 vs. UNT cells at each shear stress magnitude; **P < 0.05 vs. static TNF-α. B: IRF-1 expression in HAECs sheared in the Hele-Shaw device while simultaneously exposed to TNF-α (0.3 ng/ml) alone, TGRL (10 mg/dl ApoB) alone, or TNF-α and TGRL together for 2 h. Data are presented as percentages of static TNF-α. Values are means ± SE; \( n = 3 \). *P < 0.04 vs. TNF-α alone at each shear stress magnitude; #P < 0.05 vs. the static point for the corresponding treatment. C: nuclear translocation of NF-κB p65 was quantified in HAECs exposed to a linearly varying shear field in the presence and absence of TNF-α for 1 h. The translocation of p65 is presented as MFI per nucleus. Values are means ± SE; \( n = 3 \).
and bifurcations. Given the functional significance for VCAM-1 as a biomarker for atherogenesis whose expression lies at the convergence of dietary and hemodynamic factors, we used it as a readout of endothelial inflammation in our experiments. We have previously reported that the changes in VCAM-1 expression elicited by TGRL were IRF-1 dependent and strongly correlated with monocyte recruitment to cultured HAECs under shear flow (36, 39, 44) over a dose range in TNF-α that was significantly affected by IRF-1 suppression or overexpression (36). To shed light on the molecular mechanisms by which hydrodynamic shear stress modulates inflammation associated with hypertriglyceridemia, a custom microfluidic device was used that produces a continuous gradient in shear stress over a monolayer of HAECs, allowing visualization of the focal response to inflammation and shear stress during exposure to dietary TGRL (40). We discovered that hydrodynamic shear stress and TGRL each can regulate IRF-1 and superpose to exert transcriptional regulation of VCAM-1 expression over relatively short distances (i.e., ~100 EC diameters), corresponding to small changes in shear stress (i.e., ~2 dyn/cm²). These changes are functionally relevant in light of our previous studies (36, 40, 44) in which increased VCAM-1 expression tracked 1:1 with enhanced monocyte adhesion.

While pro-TGRL significantly amplified IRF-1 and VCAM-1 expression levels above TNF-α alone, shear stress exerted an independent influence on the degree of this enhancement that varied as a function of position in the flow channel corresponding to shear stress magnitude. Although the entire monolayer was bathed from the inlet to the outlet with the same TGRL/TNF-α-fortified media, VCAM-1 expression varied from a maximum at 2 dyn/cm², dropping ~100% at the upstream region, corresponding to 12 dyn/cm². It is noteworthy that the effect elicited by anti-TGRL did not overwhelm the propensity of low shear stress to enhance VCAM-1 expression relative to TNF-α stimulation alone. A remarkable discovery was that the modulatory effect of shear stress magnitude was abolished by siRNA suppression of IRF-1, highlighting its critical role in

![Diagram](image-url)

**Fig. 4.** TGRL- and shear stress-modulated VCAM-1 upregulation are IRF-1 dependent. HAECs were transfected with human IRF-1 small interfering (si)RNA (siIRF) or scrambled siRNA control (siCtrl). After 48 h, cells were sheared in the Hele-Shaw device with TNF-α alone (A) or in combination with pro-TGRL (B) for 4 h. Data are presented as percentages of static TNF-α transfected with siCtrl. Values are means ± SE; n = 3–4. *P < 0.04 vs. siIRF at each shear stress magnitude.

**Fig. 5.** IRF-1 expression is elevated at sites of atherosusceptibility in the porcine aortic endothelium. A: EC lysates were harvested from the inner curvature of the arch (a representative atherosusceptible site) and the descending thoracic aorta (a representative atheroresistant site) of young, disease-free swine. B: endothelial IRF-1 protein expression by Western immunoblot analysis, quantified by densitometry and normalized to GAPDH. The representative blots show matched samples from one animal. Values are means ± SE; n = 11. C and D: IRF-1 expression by immunohistochemistry in the endothelium at a representative site of atherosusceptibility (C) and atheroresistance (D) from the same animal. Arrowheads indicate positive IRF-1 staining on the luminal endothelium.
hydrodynamic modulation of VCAM-1 expression. The patterns in expression observed in our flow channel demonstrate the high spatial acuity by which ECs respond to a gradient in shear stress and are consistent with in vivo observations demonstrating prominent VCAM-1 expression at the aortic arch and virtually no expression just millimeters away (6, 37). Together, our data in cultured ECs demonstrate a causal relationship by which low shear stress signaling is synergistic with atherogenic TGRL in the transcriptional regulation of VCAM-1 production via IRF-1. This mechanism may prove relevant to the focal susceptibility to atherosclerotic plaque formation under dyslipidemia.

In light of the prominent role for VCAM-1 in atherogenesis, there has been considerable attention devoted to demonstrating its regulation by hydrodynamic shear stress in the endothelium. VCAM-1 exhibits a duality in its modulation by flow in that high-magnitude and nonreversing shear stress suppress (1, 7, 31, 34), while low-magnitude and reversing shear stress upregulate or enhance, cytokine-induced expression (4, 13, 14, 19). Although multiple laboratories have reported that shear stress can influence arterial VCAM-1 expression, an understanding of the mechanisms underlying its enhancement at low regimes of shear stress remains incomplete. It is well known that cytokine induction of VCAM-1 elicits the activity of a conserved set of transcription factors, including NF-κB and activator protein-1. The VCAM-1 promoter also contains additional regulatory elements that discriminate its expression pattern from other adhesion molecules like ICAM-1 (9, 10). Optimal VCAM-1 expression requires both constitutively bound SP1 and IRF-1, the latter of which is upregulated by TNF-α and acts cooperatively with NF-κB to induce a maximal response (29, 30). Our data suggest that regulation in the expression of IRF-1, but not NF-κB, is predominant in determining the pattern of VCAM-1 expression observed in response to postprandial TGRL and shear stress (36).

Our data support a direct role for shear stress in the regulation of IRF-1 expression, which, in turn, plays a key role in modulating the VCAM-1 responsiveness to shear. The pattern in expression of TNF-α-induced VCAM-1 mirrored that of IRF-1 along the channel, exhibiting maximal expression at 2 dyn/cm² and significant downregulation at 12 dyn/cm² compared with the static condition. Moreover, the suppression of IRF-1 with siRNA effectively lowered overall inflammatory VCAM-1 upregulation and abrogated its enhancement at low shear stress, demonstrating the specificity of IRF-1 in these responses. In contrast, the nuclear NF-κB signal was uniform along the channel and appeared insensitive to modulation by shear. As shear stress has been documented to modulate NF-κB activation (2, 9, 27), these results may be reflective in part of a transient response to flow. Our data are consistent with a previous report (27) showing that NF-κB is initially activated uniformly by the onset of flow but that its activation is sustained only under conditions of low shear stress. We conclude that while NF-κB is necessary for the cytokine-induced activation of VCAM-1, its expression at 1 h did not factor prominently into the observed shear modulation of VCAM-1. Rather, our data support a mechanism by which shear-modulated IRF-1 acts cooperatively with cytokine-induced NF-κB to tune the level of VCAM-1 expressed over short time intervals. At the low shear range, maximal levels of VCAM-1 occurred at 2 dyn/cm² where nuclear IRF-1 was upregulated within 30 min. Conversely, at high shear stress, VCAM-1 was low compared with the static region, even though NF-κB was uniformly activated, in part because IRF-1 was diminished. It is noteworthy that VCAM-1 tended to decrease further with increasing shear magnitude even in the presence of siRNA suppressing IRF-1 (Fig. 4A). This may reflect the activation by high shear stress of atheroprotective transcription factors, such as Kruppel-like factor 2 and peroxisome proliferator-activated receptor-γ, that are known to counteract the influence of NF-κB on inflammation (3, 31).

It is well published that arterial hemodynamics predisposes certain regions of the circulation to atherosclerosis; however, it is also noted that lesions do not form spontaneously in the absence of other factors that promote systemic inflammation. In the widely used ApoE-deficient mouse model of atherosclerosis, there is a strong correlation between sites of VCAM-1 expression and subsequent lesion formation, and plaque progression is greatly accelerated when animals are fed a high-fat diet (23). The preclinical swine model has also proven useful to understanding early changes in the endothelium that are relevant to atherosusceptibility. The aortic endothelium derived from representative sites of susceptibility, and resistance to atherosclerosis studied in swine revealed regional heterogeneity consistent with priming for inflammation and sensitization to pathological change at sites of flow disturbance (8, 15, 32). The emerging paradigm is that disturbed flow is permissive to atherosclerosis in that it contributes to a state of chronic stress that might be exacerbated by factors that affect systemic inflammation, causing a transition of the endothelium to a state of dysfunction that precedes pathology (12). Consistent with this view of vulnerable endothelium, our data demonstrate that IRF-1 is more highly expressed at sites of atherosusceptibility in the arteries of disease-free swine. This is an intriguing finding in that it suggests that even in the absence of inflammatory mediators or plaque formation, certain sites are predisposed to a heightened inflammatory response through basally elevated levels of IRF-1. In these same regions, NF-κB is elevated but inactive and VCAM-1 expression is not observed (32). The implication is that disturbances in arterial flow chronically prime the endothelium for a heightened inflammatory response to a subsequent challenge. Indeed, preliminary data from our laboratory in a high-cholesterol diet-fed swine model demonstrated further enhancement of IRF-1 expression combined with lipid infiltration at atherosusceptible sites after 5 wk on a high-fat diet, consistent with the initiation of fatty lesions associated with atherogenesis.

The use of flow models with cultured endothelium facilitates mechanistic studies and the demonstration of causality through the precise experimental manipulation of shear conditions. The Hele-Shaw microfluidic device in the present study provided the added advantage of enabling the quantification of outcomes associated with endothelial function and inflammation over a range of shear stress conditions in a single monolayer, using TGRL samples derived from individual subjects at quantities that would otherwise have limited observation to a single shear stress magnitude. Our approach shares the limitations of any culture model in the assumption that it will adequately recapitulate the complexity of the environment in native arteries where ECs are chronically adapted to complex flow patterns and are subject to interactions with other cells, the matrix, and humoral mediators that regulate their behavior. The present
study was limited to the investigation of laminar shear stress of constant but varying magnitude, commonly used as simple approximations of flow in arteries. Consistent with previous findings in cultured ECs, high shear stress attenuated the response to cytokine activation relative to low shear stress (6, 11, 14). Although it is possible that more complex waveforms could evoke a different endothelial response, we (14) have previously reported similar responses in TNF-α-induced VCAM-1 expression to pulsatile shear and oscillatory shear of comparable time-average values. In general, the results are consistent with a greater permissiveness to inflammatory activation of ECs at sites of flow disturbance in vivo. It is also noteworthy that the inflammatory response of HAECs to TNF-α was more than twofold higher than in human umbilical vein ECs (data not shown). Thus, the HAEC response in this vascular mimetic assay is more representative of an arterial phenotype and useful to study mechanisms associated with atherogenesis.

Our assessment of VCAM-1 after 4- or 24-h exposure to shear stress and cytokine correlated with the peak in inflammatory response to low-dose TNF-α stimulation. Atherosclerosis presents as a chronic inflammatory disease but has also been characterized as a postprandial phenomenon, affected not only by a heightened state of metabolic-induced inflammation but also exacerbated by events such as a single high-fat meal (33, 42) and promoted by repetitive exposure to transiently elevated lipoproteins and associated endothelial dysfunction (17, 36, 39, 44). The results of the present study, together with our previous reports on the inflammatory nature of TGRL, are consistent with repeated episodes of enhanced inflammation in response to dietary factors that can have long-term cumulative consequences (33, 45).

Using this reductionist approach, we demonstrated that TNF-α, TGRL, and fluid shear stress each modulated endothelial IRF-1 expression. While the underlying signaling pathways are likely to be complex and not necessarily independent, the coincidence of low shear stress and elevated IRF-1 that could be amplified by proatherogenic TGRL reveals a viable mechanism for the upregulation of VCAM-1 expression at sites predisposed to plaque formation. We propose that IRF-1 may play a role in focal atherogenicity by modulating endothelial VCAM-1 expression in response to multiple stimuli.

In summary, our data demonstrate a direct role for fluid shear stress in the regulation of IRF-1 expression by the endothelium. This mechanism explains in large part the modulation of TNF-α-induced VCAM-1 expression by shear stress. Both shear stress and postprandial TGRL exerted distinct effects that converged on IRF-1 to augment cytokine-induced VCAM-1 expression. Notably, low shear stress and TGRL from hypertriglyceridemic subjects were synergistic in promoting maximum VCAM-1 expression. These data obtained from EC monolayers cultured under a continuous gradient of shear stress indicate that IRF-1 may contribute significantly to focal atherosusceptibility by responding to both hemodynamics and dyslipidemia to modulate VCAM-1 expression in the endothelium.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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SHEAR-MODULATED IRF-1 EXPRESSION


