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Hemodynamic Regulation of Inflammation at the Endothelial-Neutrophil Interface

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Abstract

Arterial shear stress can regulate endothelial phenotype. The potential for anti-inflammatory effects of shear stress on TNFα-activated endothelium was tested in assays of cytokine expression and neutrophil adhesion. In cultured human aortic endothelial cells (HAEC), arterial shear stress of 10 dyne/cm^2 blocked by > 80% the induction by 5 ng/ml TNFα of interleukin-8 (IL-8) and IL-6 secretion (50% and 90% reduction, respectively, in the presence of nitric oxide synthase antagonism with 200 μM nitro-L-arginine methylester, L-NAME). Exposure of TNFα-stimulated HAEC to arterial shear stress for 5 hr also reduced by 60% (P < 0.001) the conversion of neutrophil rolling to firm arrest in a venous flow assay conducted at 1 dyne/cm^2. Also, neutrophil rolling lengths at 1 dyne/cm^2 were longer when TNFα-stimulated HAEC were presheared for 5 hr at arterial stresses. In experiments with a synthetic promoter that provides luciferase induction to detect cis interactions of glucocorticoid receptor (GR) and NFκB, shear stress caused a marked 40-fold induction of luciferase in TNFα-treated cells, suggesting a role for GR pathways in the anti-inflammatory actions of fluid shear stress. Hemodynamic force exerts anti-inflammatory effects on cytokine activated endothelium by attenuation of cytokine expression and neutrophil firm arrest.

Keywords

shear stress, inflammation, glucucorticoid, neutrophils

Introduction

Atherosclerosis is a chronic inflammatory disease that is often hemodynamically localized at sites of low and reversing shear stress.[1, 2] Inflammatory markers are distinct indicators in the development and the progression of atherosclerotic lesions, which are heavily infiltrated with macrophages, T-lymphocytes and other cellular components of inflammation.[3] During inflammation, the initial rolling interaction of neutrophils on activated endothelium is mediated by selectins.[4] Firm adhesion occurs when β2 integrins on neutrophils are up regulated by chemokines such as IL-8 to mediate binding to adhesion molecules (ICAM-1) on the activated endothelium.[5] Proinflammatory cytokines such as TNFα or IL-1 induce the expression of several cytokines and cell adhesion molecules by endothelial cells.[3]

With respect to steroidal anti-inflammatory drugs, there are two main mechanisms by which glucocorticoids are thought to exert their therapeutic actions in suppressing inflammatory and immune responses. Glucocorticoids diffuse into the cytoplasm and bind to glucocorticoid receptor (GR), which then translocate into the nucleus and bind as dimers to the glucocorticoid response elements (GRE) present in various promoters.[6] Examples of genes regulated through GRE in their promoters include IL-8 and IL-2 receptor α.[7] Activated GR can also suppress inflammation by directly interacting with activated transcription factors, such as nuclear factor-kappa B (NFκB) and activator protein-1 (AP-1), thus altering NFκB or AP-1 participation in inflammatory gene expression.[8, 9] In most cases, the active form of NFκB is a heterodimer of RelA (p65) and NFκB1 (p50) released from its inhibitor IκB. Steroid-liganded GR can directly interact with the p65 subunit of NFκB[10] as well as interfering with transcriptional cofactors CREB binding protein (CBP) and steroid receptor coactivator-1 (SRC-1).[11] In the endothelium, TNFα inducible genes that are down regulated by the presence of dexamethasone include:

interferon β, platelet-derived growth factor (PDGF) B subunit, transforming growth factor (TGF) β2, vascular endothelial growth factor receptor 3 (VEGFR3), IL-1, 2, 7, and 8.[12] Glucocorticoids also suppress the expression of inflammatory markers such as adhesion molecule expression (ICAM-1, E-selectin, VCAM-1)[13-15] and various interleukins. GR suppresses NFκB induction of the IL-6 gene in vascular endothelial cells,[16] indicating that the precise mechanisms of NFκB down-regulation by nuclear steroid receptors can be gene-specific since IL-6 lacks any apparent GRE in its promoter.

The endothelium also acts as a dynamic interface between biochemical triggers and mechanical factors (hemodynamics) and inflammatory cell adhesion. Various kinases activated during mechanotransduction can alter the activity of transcription factors such as NFκB, AP-1, erg-1, and GR.[17-20] A recent study by Chiu et al. showed that shear stress on endothelial cells alters TNFα-stimulated expression of ICAM-1, VCAM-1 and E-selectin while decreasing TNFα-stimulated NFκB -DNA binding activity in mobility shift assay,[21] although no tests of cytokine secretion or endothelial adhesiveness were conducted in that study. Glucocorticoid receptors are present in endothelium and smooth muscle cells.[22, 23] In our prior study, we demonstrated that shear stress caused endothelial GR nuclear localization and activated transcription from a GRE promoter through pathways sensitive to inhibitors of the shear-activated kinases, MEK1/2 kinases and PI-3 kinase.[20] This finding suggests certain parallels between the atheroprotective role of unidirectional shear stress and the anti-inflammatory actions of the GR. We now test the hypothesis that shear stress is anti-inflammatory, specifically in suppressing TNFα-induced endothelial activation with respect to the expression of secreted cytokines and neutrophil adhesion.

Materials and Methods

Cell Culture and Reagents

Human aortic endothelial cells (HAEC) were maintained in EGM-2 endothelial media system (Clonetics). Glass slides were coated with type I collagen (BD Biosciences). For flow chamber experiments, cells were seeded on collagen coated 38 x 75 mm glass slides at a density of 1 to 2 x 10^6 cells per slide and cultured to confluency. TNFα was obtained from Sigma. Incubated cell culture media from HAEC was collected and measured for cytokine content using human IL-8 and IL-6 ELISA immunoassays (R & D Systems) according to the manufacturer's instructions. IL-8 and IL-6 concentrations were used to calculate the total amount of cytokine produced after accounting for volume changes, and normalized with respect to the total number of cells in each experimental group.

Neutrophil Isolation

Human blood was collected from healthy adult donors by venipuncture and anticoagulated with Na-citrate (9 parts blood to 1 part Na-citrate) and neutrophils were isolated over neutrophil isolation medium (Robbins Scientific) as previously described.[24] After isolation, neutrophils were resuspended in Hank's balanced salt solution (HBSS, Gibco Laboratories) supplemented with 2% HBS, counted, and diluted to a final concentration of 0.75 x 10^6 cells/ml.

Shear Stress Exposure and Neutrophil Adhesion Studies

Cells were exposed to laminar shear stress in parallel plate flow chambers attached to flow loops for media recirculation (15 ml) in a 37°C incubator as previously described.[20] Wall shear stress was calculated as: $\tau_{wall} = 6\mu Q/bh^2$ for viscosity, μ = 0.01 dynes-sec/cm^2; Q, volumetric flow rate (cm^3/s); b, flow chamber width (2.5 cm); h, the total plate separation (0.025

cm). For neutrophil adhesion studies, following 5 hr arterial shear stress exposure at 10 dyne/cm^2 ($\pm$ 5 ng/ml TNFα), the flow chambers were reconnected to a Harvard syringe pump for infusion of a neutrophil suspension at wall shear stress of 1 dyne/cm^2. During the neutrophil adhesion studies at venous flow conditions, flow chambers were imaged by phase contrast microscopy (Zeiss Axiovert 135, 20X Plan Apochromat) and recorded on videotape for subsequent digital image analysis. Neutrophils were perfused over HAEC for 5 min before the start of image acquisition. Each field of view (FOV; 0.1 mm^2) of neutrophils flowing over HAEC was recorded in 10-sec video segments from which total and firmly adherent neutrophil counts were determined. "Firm adhesion" refers to neutrophils that remained stationary during 10 seconds, and "total" refers to average number of neutrophils that interacted with the endothelial monolayer in the FOV over the 10-sec interval. Rolling distance was generated using the multi-tracking function of ImageJ (NIH).

Promoter constructs

The pGRED was kindly provided by Dr. Alexander Whitehead (U. Penn.) [25]. The pGRED contains the SAA2 promoter with a deletion of a 9-basepair interruption of the GRE consensus sequence, thus providing an active GRE and an active NFkB site in the promoter upstream of firefly luciferase. *Renilla* luciferase transfection control plasmid was from Promega. For dual luciferase assays, endothelial cultures were washed in PBS and lysed in Passive Lysis Buffer (Promega). Lysates were assayed for luciferase and *Renilla* activity using the LAR II and Stop and Glo Reagents (Promega) in a dual-injection luminometer.

Results

Shear stress attenuates TNFα induced IL-8 and IL-6 secretion

Addition of 5 ng/ml TNFα under static conditions induced a marked 45-fold increase ($P < 0.001$, n = 3) in the total amount of IL-8 secreted by HAEC in 8 hr compared to static control (Figure 1A). However, shearing the cells during TNFα exposure blocked this IL-8 secretion by 80% ($P < 0.001$, n = 3). Shear stress alone caused a small increase relative to static culture of IL-8 secretion from 1 to 3 ng/10^6cells at 8 hr. This small up-regulation of IL-8 by flow alone was not seen in the presence of LNAME (Figure 2A) since LNAME-treated cells maintained in static culture produced about 4 ng of IL-8 per 10^6 cells at 8 hr.

With respect to IL-6 secretion, adding TNFα to cell culture media induced a striking 33-fold increase ($P < 0.01$, n = 3) over static control that was reduced significantly by 90% ($P < 0.01$, n = 3) by flow (Figure 1B). Shear stress alone caused an increase of IL-6 secretion at 8 hr compared to static control from 0.05 to 0.25 ng/10^6cells that was not seen in the presence of LNAME (Figure 2B). These data demonstrated that shear stressed endothelium, when compared to stationary cultures, were considerably less responsive to TNFα with respect to IL-8 and IL-6 secretion.

Either TNFα receptor mediated signaling proximal of NFκB activation was disrupted in sheared cells and/or shear stress triggered factors, e.g. nitric oxide (NO), that antagonized NFκB function. To test the role of shear induced NO production[26] on TNFα stimulation of HAEC, we used an eNOS inhibitor nitro-L-arginine methylester (L-NAME) in conjunction with TNFα and shear stress (Figure 2). HAEC were pretreated with the L-NAME (200 μM, 1 hr), a concentration known to block shear induced NO release.[27] HAEC preconditioned in static culture or shear condition (10 dynes/cm^2, 1 hr) in the presence of L-NAME, were then

maintained further in the presence or absence of TNFα (5 ng/ml). In comparing the static control groups from Figure 1 and Figure 2, there was a small increase of basal IL-8 secretion and a marked increase in IL-6 secretion by HAEC cells pretreated with L-NAME, indicating that the basal production of NO by static cells limited IL-8 and IL-6 expression. In static HAEC cultures, pretreatment with L-NAME did not prevent the marked increase of IL-8 and IL-6 secretion by TNFα, and again shearing in media with TNFα yielded a significant reduction of cytokine expression (50% for IL-8 and 90% for IL-6). With L-NAME present, shear stress reduced IL-6 production in TNFα-stimulated cells to levels below the matched static control cultures. These data indicate that shear stress interfered with TNFα-induced increase of IL-8 and IL-6 without a strict requirement for flow-induced NO.

Shear stress attenuates neutrophil firm arrest on TNFα-activated HAEC

HAEC monolayers were treated with 5 ng/ml TNFα in the presence or absence of arterial shear stress for 5 hr before a neutrophil adhesion assay at 1 dyne/cm^2. Time averaged images (Figure 3) allowed detection of rolling and arrested neutrophils. On control cultures without TNFα, neutrophils passed over the endothelial surfaces with essentially no rolling or arrest. HAEC exposed to shear stress for 5 hr alone (no TNFα) did not promote neutrophil adhesion, indicating that shear stress alone was not pro-adhesive. HAEC cells treated with TNFα, on the other hand, were strongly activated with about 38.61 ± 6.92% (n = 15 FOV) of interacting neutrophils becoming firmly arrested (Figure 4A). Cells maintained under arterial shear stress for 5 hr during the TNFα induction, however, had 60% ($P < 0.001$) fewer neutrophils converting to firm arrest (11.7 ± 4.59% of all interacting neutrophils, n = 15 FOV). The total number of neutrophils (rolling and arrested) that came to interact with shear stressed HAEC plus TNFα

versus static HAEC treated with TNFα was not significantly different (56.1 ± 18.8 versus 46.3 ± 17.9 per FOV).

To further quantify the neutrophil interactions with endothelial surfaces at 1 dyne/cm^2, the rolling length in microns over a 10-sec interval was determined. The results are presented in Figure 4B, in the form of a histogram, generated on the tracked movement of 188 and 212 neutrophils over static and sheared HAEC, respectively. On TNFα-stimulated HAEC in the absence of arterial shear stress exposure, most neutrophils had short rolling lengths (90% < 21 μm) with the longest being 37 μm and with a median length of 8.83 μm. On sheared HAEC treated with TNFα, however, the rolling length increased up to 113 μm with the median length of 24 μm. The mean rolling length for both cases was also significantly different: 10.6 μm (no shear) versus 30.1 μm (10 dyne/cm^2) ($P < 0.001$).

Interactions between glucocorticoid receptor and NFkB in sheared endothelium

While disturbed hemodynamics may enhance endothelium susceptibility to atherosclerosis, we have detected the anti-inflammatory effects of unidirectional arterial shear stress in attenuating TNFα-activated endothelial cytokine production (Figures 1 and 2) and neutrophil adhesion (Figures 3 and 4). The net effect of shear stress, however, encompasses a variety of transcriptional factors such as AP-1, SP-1, and GR [17-20] to potentially regulate NF-κB function in a promoter specific manner. We sought to investigate possible interactions between shear stress activated GR functions, independent of dexamethasone [20], on NFκB function. To detect *cis* interactions between GR and NFκB on a promoter, we employed an artificial promoter construct that is induced when GR and NFκB bind the promoter. The wildtype SAA2 promoter contains binding sites for NFκB, AP-1, and NF-IL6, along with a disrupted GRE site [28]. The inactive GRE sequence is interrupted in the middle by a function-blocking 9-bp insertion.

Removal of this 9-bp insertion renders the promoter responsive to dexamethasone potentiation (Figure 5A) in the presence of cytokine stimulation [25]. This artificial promoter construct based on the SAA2 deletion (pGRED) is unique in that, when induced by a cytokine, its transcriptional activity is *enhanced,* not repressed, by dexamethasone. This allows "light-up" detection of NFκB-GR cross talk. While dexamethasone typically down regulates NFκB function, this may occur via GR binding to NFκB, either on or off the promoter. Because GRED involves dexamethasone potentiation of NFκB, the GRED construct allows detection of GR modulation of activated NFκB on the promoter.

TNFα (5 ng/ml) caused a 15-fold induction of GRED which was further enhanced by dexamethasone (10 pM) (Figure 5B). The GRED promoter was not responsive to dexamethasone alone, consistent with the enhancer function of GR when in the presence of NFκB on the promoter. Applying shear stress alone activated GRED, thus detecting activation of both GR and NFκB by shear stress. The combination of TNFα with shear stress caused a striking 40-fold elevation of transcriptional activity from GRED. Taken together, these data suggest that having the intact GRE sequence present renders the GRED promoter highly responsive to shear stress, particularly in the presence of strong NFκB functionality in TNFα-stimulated endothelium.

Discussion

Shear stress activation of GR receptor and GRE transcriptional regulation[20] provides a mechanism for potential cross talk between mechanotransduction and anti-inflammatory actions. In this study, we demonstrated that shear stress at 10 dynes/cm^2 attenuated TNFα-stimulated IL-6 and IL-8 expression in cultured human endothelial cells (Figures 1 and 2). Shear induced inhibition of TNFα-stimulated IL-6 and IL-8 expression did not strictly require the presence of NO (Figure 2), which suggests that the anti-inflammatory actions of shear stress is independent of its vasodilatory effects through stimulated NO release. We have previously shown that pretreating endothelial cells with L-NAME had no effect on shear induced GRE-SEAP promoter construct activation at 6 hr, as a metric of shear activation of endogenous GR function. The anti-inflammatory effect of shear stress was also apparent in neutrophil-endothelial interactions. Endothelial monolayers exposed to arterial shear and static endothelium respond differently to TNFα stimulation, as evidenced by more sustained neutrophil rolling (longer rolling length) and less conversion to firm arrest on cells pre-exposed to 10 dyne/cm^2 (Figure 3 and Figure 4). A likely explanation for the increased rolling length could be the decreased expression of IL-8 and/or reduced presentation of ICAM-1 or VCAM-1 by sheared endothelial cells. The GRED promoter construct was designed to provide a "light-up" signal to detect GR-NFkB interactions at the level of a promoter. Shear stress proved to be a particularly strong inducer of the GRED promoter in TNFα-stimulated endothelium.

Previous research has shown that IL-8 in solution rapidly induces rolling neutrophils to arrest. Also, increasing immobilized IL-8 decreases neutrophil rolling distance and promotes firm adhesion.[29] IL-8 in the fluid phase or bound to endothelium glycoaminoglycan may increase β2-integrin avidity, leading to neutrophil firm arrest through ICAM-1/β2 integrin

interactions.[5] Sheared endothelial cells express less IL-8 upon cytokine stimulation (Figure 1A), which may lead to less neutrophil firm adhesion and longer rolling lengths. Chiu et al showed decreased DNA binding activity of NFκB in cells that were exposed to shear stress in addition to TNFα.[21] This finding of NFκB down regulation is quite consistent with the reduced IL-6 and IL-8 expression in sheared HAEC stimulated with TNFα that we demonstrated. In fact, this decreased IL-6 and IL-8 presentation may contribute significantly to altered interactions between neutrophils and sheared endothelium since its mechanism is independent of NO inhibitor. On the other hand, NO inhibitor has been shown to abolish the attenuating effect of shearing on elevated endothelial VCAM-1 expression induced by TNFα and lipopolysaccharide.[30] Finally, we also saw that shearing endothelial cells alone, in the absence of cytokine stimulation, does not promote neutrophil adhesiveness, possible because shear stress alone does not substantially induce expression of VCAM-1 or ICAM-1 on HAEC.[31]

While increased P-selectin or E-selectin facilitates neutrophil rolling and ICAM-1 or VCAM-1 expression aids in neutrophil firm adhesion, our findings in Figure 4A suggest that shear stress influenced only the conversion to firm arrest since total interacting neutrophils (rolling and arrested) were the same regardless of preshearing. Prior studies have investigated various aspects of endothelial response to stimulation by TNFα in the presence of shear stress, although none have previously measured endothelial adhesiveness to human neutrophils. Yamawaki et al [32] showed in an ex vivo model of rabbit aorta that shear stress inhibited TNFα stimulated VCAM-1 expression. Chiu et al[21] also demonstrated in human umbilical endothelial cells that that stress decreases TNFα-induced VCAM-1and E-selectin expression, while enhancing TNFα-induced ICAM-1 mRNA and protein expression. It is difficult to predict the net effect on neutrophil adhesion from these two prior studies since E-selectins, ICAM-1 and

VCAM-1 are being altered in differing ways. Additionally, the membrane-cytoskeletal structure function is likely altered in endothelial cells during shear stress exposure and this may have subsequent effect on bond life, independent of receptor number due to changes in membrane extension and tethering. The VCAM-1 data from these studies correlates well with the reduced neutrophil interactions that we saw. No change in net rolling, a selectin-mediated process, was observed in our measurements due to preshearing of the TNFα-stimulated endothelium. On the other hand, ICAM-1 levels under shear has been consistently observed to be different from VCAM-1 or E-selectin,[21, 33-35] and these studies suggested that both NFκB transcriptional activation and oxidative stress (reactive oxygen species) differentially influence TNFα induced secretion of cytokines and adhesion molecules. The ICAM-1 promoter region contains binding sites for AP-1, SP-1 and NFκB, rendering its expression sensitive to regulation by a number of transcriptional factors under both shear stress and cytokine stimulations.[36] The effect of arterial shear stress on TNFα activation of endothelium has been addressed before in previous studies.[32] However, this is the first functional assay of direct neutrophil-endothelial interactions under both TNFα and shear stress stimulation. Though previous studies have presented data on expression levels of adhesion molecules, there were no direct measurements of altered endothelial adhesiveness toward neutrophils. This is the first study to measured alteration of neutrophil rolling on TNFα-stimulated endothelium due to pre-exposure to arterial shear stress.

In an experiment of this type, LNAME may have regulatory effects on baseline properties of endothelium, effects on TNFα-stimulated properties, and effects on mechanobiological responses via NO in the presence or absence of TNFα. For example, LNAME is known to have unexpected additional actions on endothelium beyond the inhibition of NO production[37] since NO is active as an autocrine agent. We note that LNAME reduced IL-8 production by TNFα-stimulated endothelium under no-flow conditions. In contrast, LNAME on its own (without TNFα or flow) enhanced IL-6 production indicating

an additional role of LNAME on IL-6 regulation not seen for IL-8. Complex autocrine loops regulating the IL-6 and IL-8 genes may become unmasked with the use of chemical inhibitors such as LNAME and this is seen with IL-6 which was up-regulated by the use of LNAME on its own. Still, shear stress markedly reduced TNFα-stimulated IL-8 and IL-6 production as seen in Fig. 1 and 2.

The anti-inflammatory effect of shear stress on TNFα activation is further supported by microarray studies of endothelial gene expression.[38] As microarray studies of gene expression became more sophisticated, a recent study analyzed differential changes in endothelial transcription profiles of disturbed versus undisturbed laminar flow regions of the same pig aorta.[39] Proinflammatory adhesion molecules such as VCAM-1, ICAM-1, E-selectin, P-selectin were not differentially expressed in these regions, while IL-6 and IL-8 receptor β are up-regulated in disturbed regions and IL-8 is slightly down-regulated in disturbed regions. These data are in good agreement with our data on the attenuating effect of elevated shear stress on IL-6 and IL-8 expression. Taken together, these data suggest that interleukins (IL-6, IL-8) display increased sensitivity toward varying flow conditions, and changes in their expression may be a precursor to altered presentation of inflammatory adhesion molecules.

As an initial step toward studying shear activated GR and NFκB function, we studied the interaction between shear activated GR and NFκB at their corresponding promoter sites, utilizing a modified SAA2 promoter constructs that presents binding sites for AP-1, SP-1, and a functional GRE sequence resulted in overall increased activation of reporter gene. It should also be noted that binding of shear induces and activates fos/jun (the AP-1 complex) which can also antagonize NFκB function. Shear stress activated GR pathway, independent of dexamethasone, may interfere with cytokine enhanced NFκB functions in inflammation. However, the overall effect of shear stress encompasses a variety of transcriptional factors, including AP-1, SP-1, and NF-κB that may interfere with GR transcriptional functions as well.

TNFα is a strong activator of NFκB,[40, 41] a key transcription factor in the up-regulated expression of inflammatory markers including IL-6 and IL-8.[42, 43] Previous studies have shown that NFκB mediated interleukin expression can be repressed by ligand activated GR,[9, 44] suggesting that shear stress may also exert its inhibitory effects against TNFα through the activation of GR and GRE pathway. The atheroprotective effects of shear stress on the endothelium may be attributed to various anti-inflammatory processes, along with its ability to modulate the release of vasoactive factors such as NO, prostacyclin, endothelin-1, MCP-1, and vascular epidermal growth factor (VEGF).[45-48] Suppressing inflammation could be a key mechanism by which shear stress exerts its atheroprotective functions in the endothelium. Recent analysis of gene expression profile in normal pig aorta revealed that the endothelium in disturbed flow region is primed for inflammation[39] where genes for several general pro-inflammatory cytokines and receptors such as interleukin 1α, IL-1 receptor 1, IL-6, IL-8 receptor β, and monocyte chemotactic protein 1 (MCP-1) are up regulated compared to laminar flow area. However, the NFκB system is primarily inactivated, consistent with the unaffected expression of inflammatory cells adhesion molecules between two flow regions. Thus, while varying hemodynamics may alter endothelium liability to atherosclerosis, we presented data supporting the anti-inflammatory effects of shear stress in inhibiting TNFα activated endothelial activation and neutrophil interactions. The net effect of shear stress encompasses a variety of transcriptional factors such as AP-1, SP-1, and GR to regulate NFκB function in a promoter specific manner.

Acknowledgements

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Figures

Figure 1: Effect of shear stress on TNFα activated expression of cytokines IL-8 (A) and IL-6 (B) in HAEC.

Cells were cultured and maintained under static condition (Cont) or treated with TNFα, 5 ng/ml, for 8 hrs (TNFα). HAEC were pretreated with shear stress at 10 dynes/cm^2 for 1 hr before TNFα was injected into flow media without interruption. Cells were sheared in either the presence or absence of TNFα media for 8 hr. Data are presented as mean ± SE (n = 3). * $P <$ 0.01 and ** $P < 0.001$ refer to significant difference compared to static TNFα alone.

Figure 2: Effect of L-NAME on shear attenuation of TNFα induced IL-8 (A) and IL-6 (B) secretion in HAEC.

Cells were pretreated with the eNOS inhibitor L-NAME (200 μM) for 1 hr. Static cells were maintained in media containing L-NAME and treated with TNFα (5 ng/ml) for 8 hr. Sheared cells were further pre-conditioned at 10 dynes/cm^2 for 1 hr in L-NAME media before TNFα was injected and sheared for another 8 hr. Data are mean ± SE (n = 3 in each group). * P < 0.005, ** $P < 0.001$, refers to significant difference compared to static TNFα alone.

Figure 3: Neutrophil adhesion studies on HAEC monolayers.

Static HAEC cultured on glass slides are treated with either media or TNFα (5 ng/ml) for 5 hr before neutrophil assays. Sheared HAEC on glass slides are exposed to shear stress at 10 dynes/cm^2 for 5 hr in media with or without TNFα before assaying with neutrophils. One-second image sequences were captured from 10-second video segments, and processed to generate the time-sequence images.

Figure 4: Total and firmly adhered neutrophil over TNFα treated sheared and static endothelial cells are quantified in (A) for 5 different images in each treatment.

Data are presented as mean ± SE (n = 5). ***** $P < 0.001$, refers to significant difference in firm adhesion between TNFα treatment of HAEC under static or shear stress conditions. Finally, rolling length for 188 and 212 neutrophils over static and sheared HAEC, respectively, are generated and presented in a histogram **(B)**. In each case, neutrophils are collected from 5 different FOVs.

Figure 5: cis-Interactions of GRE and NFkB in sheared endothelium.

(A) Alignment of the region of SAA2 and GRED promoters encompassing the GRE sequence, as compared to a consensus GRE sequence. GRED carries an intact GRE sequence following a 9-basepair deletion (Δ) from the SAA2 promoter. (B) BAEC transfected with GRED plasmid with the Renilla control plasmid are maintained in medium only, 10 μM dexamethasone, 5 ng/ml TNFα, dexamethasone with TNFα, shear stress alone (10 dynes/cm2, dpc) or shear stress and TNFα. Cells were harvested after 5 hr treatment and relative luciferase values were quantified. Data are presented as mean + SE (n = 3). * P = 0.05, * * P < 0.005, refers to significant difference between treatments with shear stress alone and shear stress with TNFα.

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