Vascular Endothelial Cells Respond to Spatial Gradients in Fluid Shear Stress by Enhanced Activation of Transcription Factors

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Abstract—The vascular endothelium is exposed to a spectrum of fluid mechanical forces generated by blood flow; some of these, such as fluid shear stress, can directly modulate endothelial gene expression. Previous work by others and in our laboratory, using an in vitro uniform laminar shear stress model, has identified various shear stress response elements (SSREs) within the promoters of certain endothelial genes that regulate their expression by interacting with various transcription factors, including nuclear factor-κB (NF-κB), early growth response-1 (Egr-1), and activator protein-1 (AP-1, composed of c-Jun/c-Jun and c-Jun/c-Fos protein dimers). In the current study, we have examined the topographical patterns of NF-κB, Egr-1, c-Jun, and c-Fos activation in a specially designed in vitro disturbed laminar shear stress model, which incorporates regions of significant spatial shear stress gradients similar to those found in atherosclerosis-prone arterial geometries in vivo (eg, arterial bifurcations, curvatures, ostial openings). Using newly developed quantitative image analysis techniques, we demonstrate that endothelial cells subjected to disturbed laminar shear stress exhibit increased levels of nuclear localized NF-κB, Egr-1, c-Jun, and c-Fos, compared with cells exposed to uniform laminar shear stress or maintained under static conditions. In addition, individual cells display a heterogeneity in responsiveness to disturbed flow, as measured by the amount of NF-κB, Egr-1, c-Jun, and c-Fos in their nuclei. This differential regulation of transcription factor expression by disturbed versus uniform laminar shear stress indicates that regional differences in blood flow patterns in vivo—in particular, the occurrence of spatial shear stress gradients—may represent important local modulators of endothelial gene expression at anatomic sites predisposed for atherosclerotic development. (Arterioscler Thromb Vasc Biol. 1999;19:1825-1834.)

Key Words: disturbed flow ■ nuclear factor-κB ■ early growth response-1 ■ c-Jun ■ c-Fos

The anatomically localized pattern of the earliest lesions of atherosclerosis strongly implicates hemodynamic forces in this disease process.1 In particular, atherogenesis occurs most frequently in association with arterial geometries with similar flow patterns, including branch points, bifurcations, and curves.2 By surgically modifying vessel configurations and thus altering local fluid mechanics in animal models, several groups have induced arterial lesions, further supporting the role of hemodynamics in disease development.3-5 During the past two decades, detailed fluid mechanical analyses of atherosclerosis-susceptible vascular geometries have revealed that these anatomic locations are exposed to a unique flow pattern identified as disturbed laminar shear stress.6-8 This shear stress regime is characterized by regions of flow separation, recirculation, reattachment, and perhaps most importantly, significant temporal and spatial gradients of shear stress.9-10 Several in vivo morphometric studies, both in native and surgically altered vessels, have demonstrated that endothelial cell structure is modified in regions of disturbed flow, with the cells displaying polygonal, nonoriented shapes, in contrast to the elongation and alignment of endothelial cells in regions of primarily unidirectional flow.11-13 Given the recognized role of endothelial cells in the atherosclerotic process, considerable attention has recently been focused on the potential role of hemodynamically induced alterations in endothelial function.14 In an attempt to specifically investigate the effects of spatial gradients in shear stress on endothelial cell biology, DePaola et al15,16 developed an in vitro model system that generates large gradients in shear stress over the relatively small dimensions of a cultured endothelial monolayer, which mimics the spatial pattern of flow separation (with reversal), reattachment, and flow recovery associated with arterial bifurcations. Utilizing this in vitro spatial disturbed flow system, previous studies in our laboratory have documented flow-specific endothelial shape changes and also have demonstrated that cell migration...
and proliferation are enhanced by disturbed flow compared with uniform laminar shear stress.\textsuperscript{15–17} However, systematic, quantitative analyses of endothelial functional changes within this disturbed flow system have been limited, in part because of the complications arising from regional differences in cell migration and proliferation, which are induced by the spatial shear gradients over the time-frame of several hours to days.\textsuperscript{16,17} In contrast, various uniform laminar shear stress systems have been extensively used to investigate hemodynamically induced alterations in endothelial function relevant to atherogenesis and, in particular, the genetic regulatory events underlying those processes.\textsuperscript{18,19}

Previous work in our laboratory utilizing a uniform flow model has identified a 6-bp (GAGACC) cis-acting transcriptional regulatory element, called the shear stress response element (SSRE), within the promoter of the platelet-derived growth factor-B (PDGF-B) gene that is required for its upregulation in endothelial cells exposed to uniform laminar shear stress.\textsuperscript{20} Subsequent studies revealed that nuclear factor-κB (NF-κB), a heterodimeric DNA-binding transcription factor, could functionally interact with the SSRE within the PDGF-B promoter.\textsuperscript{21} Recent work in our laboratory and others have identified several additional SSREs that mediate the uniform laminar shear stress induction of other endothelial cell genes by binding certain other transcription factors. Among these, the shear-induction of the platelet-derived growth factor-A (PDGF-A) gene was shown to require functional interactions between the early growth response-1 (Egr-1) transcription factor and its DNA binding sequence in the PDGF-A promoter.\textsuperscript{22} In addition, activator protein-1 (AP-1), a transcription factor composed of protein dimers of c-Jun and c-Fos, mediates the upregulation of the monococyte chemotactic protein-1 (MCP-1) gene via interactions with a nonconsensus TPA (tetra-decanoyl phorbol acetate) response element (TRE) within the promoter of this gene.\textsuperscript{23} AP-1 also mediates the downregulation of vascular cell adhesion molecule-1 (VCAM-1) through 2 consensus AP-1 binding sites.\textsuperscript{24}

Given the diversity of molecular mechanisms by which these various SSREs and associated transcription factors exert their regulatory effects, and the critical roles that they appear to play in endothelial gene regulation by uniform laminar shear stress, we chose to examine the expression patterns of NF-κB, Egr-1, c-Jun, and c-Fos in our in vitro spatial disturbed flow model. Quantitative image analysis of the nuclear localization of these rapidly activated proteins allows for measurement of an early genetic regulatory event, before significant cellular migration and proliferation can occur, and also provides biologically relevant information because the primary site of action of these proteins is in the nucleus. Our observations provide the first evidence that exposure of endothelial cells to spatially disturbed laminar shear stress, compared with uniform laminar shear stress, leads to enhanced nuclear localization of NF-κB, Egr-1, c-Jun, and c-Fos—transcription factors that are capable of functionally interacting with known SSREs. These findings provide new insights regarding the nature of pathophysiologically relevant shear stress stimuli that may participate in endothelial gene regulation at atherogenic loci in vivo.

Methods

Cell Culture

Each primary HUVEC culture was derived from a single normal umbilical cord by the method described previously.\textsuperscript{25} Single cord cultures were used because they were found to display more homogeneous staining patterns for endothelial-expressed molecules than did mixed cord cultures.\textsuperscript{26} For experimental use, second passage cells were replicate plated on multiple tissue culture-treated polystyrene coverslips (Costar Corp; Lectro Engineering Co) coated with 0.1% gelatin (Difco Laboratories Inc) and grown to confluence in Medium 199 (with 25 mmol/L HEPES; Gibco BRL) supplemented with 20% FBS (Gibco BRL), 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL endothelial cell growth supplement (ECGS; Collaborative Research Inc), and 100 μg/mL heparin (Sigma Chemical Co). After 48 hours, when confluent monolayers had formed, coverslips were transferred to a quiescence medium—Medium 199 supplemented with 5% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (no ECGS or heparin), for at least 16 hours before, as well as during, exposure to shear stress or static (no flow) conditions.

Shear Stress Apparatus

The cone-and-plate flow apparatus used to expose cultured endothelial monolayers to defined fluid shear stresses has been described in detail previously.\textsuperscript{27,28} The essential components consist of a stainless steel cone rotating over a stationary base plate that supports eight 12-mm diameter polystyrene coverslips. Culture medium was present between the cone and base plate and fresh medium was gradually exchanged (0.4 mL/min) without recirculation during the course of the experiment. The entire apparatus was maintained at 37°C in a humidified 5% CO₂, 95% air atmosphere.

The fluid mechanics of this system are described by the dimensionless parameter, $R\tilde{\nu}$:

$$R\tilde{\nu} = \frac{r^2 \omega \alpha^2}{12 \nu},$$

where $r$ is radial location on the plate, $\omega$ is angular velocity of the cone, $\alpha$ is cone angle (°), and $\nu$ is kinematic viscosity of the medium. The flow was laminar for $R\tilde{\nu} \ll 1$.\textsuperscript{28} Each tissue culture–treated plastic coverslip was glued into a stainless steel ring which contained a protuberance, in the form of a rectangular bar, that disturbed the flow immediately downstream from the bar, as described by DePaola.\textsuperscript{12} Further downstream, uniform laminar shear stress was reestablished. The bar on each ring was aligned in the radial direction, and with this configuration, nearly concentric flow had to be created so that the streamlines would strike the bar at a perpendicular angle. Deviations of up to 2° from perpendicular were considered acceptable, which corresponds to $R\tilde{\nu} = 0.0625$, as measured by Sdogoos et al.\textsuperscript{29} To achieve physiologically relevant shear stress levels, given this constraint on $R\tilde{\nu}$, it was necessary to increase the viscosity of the medium by the addition of dextran (MW 510 000; 6% wt/vol; Sigma Chemical Co), which was added to the medium contained within the cone-plate apparatus, as well as to static coverslips incubated in parallel and not exposed to flow. The resultant viscosity was measured with a coaxial cylinder viscometer at 37°C (Haake, Berlin, Germany). The position of the reattachment point, $L$ (mm), was approximated by:

$$L = (0.074) Re + 0.295$$

for $2 < Re < 7$, where $Re$, the local Reynolds number, was determined by:

$$Re = \frac{\rho \omega r^2}{\mu H},$$

with $\omega$, $r$, and $\nu$ defined as above, $h$ being the bar height (0.4 mm), and $H$ the distance between the cone and plate at position $r$ (DePaola, personal communication, 1993). For the experiments reported here, fluid mechanical parameters (cone angle, cone rotation, and medium viscosity) were adjusted such that endothelial monolayers in the uniform flow region were subjected to shear stress of 12 dynes/cm².
and the reattachment point was positioned 0.55 mm downstream from the bar (see Figure 1).

**Immunofluorescence**

After static (no flow) incubation or removal from the shear stress apparatus, cells on coverslips were rapidly rinsed in ice-cold PBS then fixed in 2% paraformaldehyde in PBS at 4°C for 20 minutes. Shear stressed coverslips were then removed from the disturbed flow rings. All of the fixed monolayers were then permeabilized in Nonidet P-40 (0.1% in PBS, 1 minute; Sigma Chemical Co), preincubated in a blocking solution of 10% goat serum, 3% bovine serum albumin, and 0.1% glycine (in PBS, 30 minutes), then incubated overnight (15 to 18 hours) with rabbit polyclonal antibodies specific for one of the human transcription factors being studied (Egr-1, NF-κB [p65], c-Jun, c-Fos; 1 μg/mL of affinity-purified immunoglobulin (IgG) in PBS plus 2% bovine serum; Santa Cruz Biotechnology). Next, the cells were successively incubated in biotinylated goat anti-rabbit IgG (15 μg/mL in PBS plus 2% bovine serum; Santa Cruz Biotechnology), followed by a Texas Red Avidin D (15 μg/mL in PBS; 45 minutes; Vector Laboratories, Inc), Texas Red Alexa Fluor 594 (45 minutes; Molecular Probes), and a Texas Red fluorophore and counterstained with the nuclear marker DAPI, as described in Methods. For each image field, a digitized fluorescent image of each dye was acquired, and a nuclear mask was created from the DAPI image and superimposed on the Texas Red image. Average nuclear fluorescence for NF-κB (p65) protein was then determined from the resultant masked image.

**Image Analysis System**

Fluorescent images were acquired and analyzed with the Oncor Image software package (Oncor, Inc) together with acquisition hardware mounted on a Nikon Microphot-FXA upright microscope (Nikon Corporation). Stained endothelial monolayers were imaged through a 20× Plan Apo objective (Nikon Corporation), fluorescently illuminated through a computer-controlled filter wheel and shutter (Ledu Electronic Products Ltd), and images were digitized via a 12-bit cooled CCD SenSys camera (Photometrics). The position of the imaged endothelial cells in relation to the disturbed flow field was determined with the aid of an XYZ motorized microscope stage (Ledu Electronic Products Ltd). All acquisition and analysis was processed by a Power Macintosh 8500/120 configured with 64 MB RAM and a Radius ThunderColor graphics interface card (Radius, Inc).

**Results**

**Quantitative Image Analysis Strategy**

Because NF-κB, Egr-1, c-Jun, and c-Fos can perform their functions as DNA-binding proteins only when in the nuclear compartment of the cell, we developed image analysis algorithms to quantitatively determine both the total amount and the cellular variability of nuclear immunohistochemically localized protein in defined regions of the endothelial monolayer within our spatially disturbed flow model. Computerized analysis was accomplished using digitized immunofluorescent images of the endothelial monolayers (280 μm in each dimension, containing ~70 cells per image). Up to 3 rows of 10 image fields each were acquired on each coverslip, positioned perpendicular to the bar on each shear stressed coverslip, and randomly positioned on each static coverslip (Figure 2A). Two static and 2 shear stressed coverslips were analyzed for each experiment. Regions of subconfluent monolayers and areas near the edge of a coverslip were excluded. The reattachment point in the disturbed flow field (near the region of maximal shear stress gradient) was at the junction between the second and third image field downstream from the bar (0.55 mm), and uniform flow was reestablished by the seventh image field (1.68 mm).

For each image field, 2 fluorescent images were acquired: a Texas Red image, indicating the amount and cellular

![Figure 1](image1.png)

**Figure 1.** The spatially disturbed laminar shear stress model. (A) Disturbed laminar shear stress patterns (including flow reversal, reattachment point, and reestablishment of uniform laminar shear stress) were created within the cone and plate apparatus by positioning a bar in the flow field.15 (B) Detailed characteristics of the downstream flow, including spatial variations in shear stress magnitudes and shear stress gradients, for the experiments described here (Re=3.4, shear stress in the downstream uniform flow region=12 dynes/cm²) were determined previously by numerical analysis (adapted from DePaola15).

![Figure 2](image2.png)

**Figure 2.** Image analysis strategy for quantitation of nuclear localization of transcription factor proteins. (A) Endothelial monolayers grown on coverslips were exposed to disturbed laminar shear stress that developed immediately downstream of a bar, as described in Methods. After fixation and staining, microscopic images were acquired at multiple positions relative to the bar. (B) In this example, HUVEC monolayers were stained for NF-κB (p65) protein using a secondary antibody coupled to a Texas Red fluorophore and counterstained with the nuclear marker DAPI, as described in Methods. For each image field, a digitized fluorescent image of each dye was acquired, and a nuclear mask was created from the DAPI image and superimposed on the Texas Red image. Average nuclear fluorescence for NF-κB (p65) protein was then determined from the resultant masked image.
localization of the transcription factor being studied, and a DAPI image, delineating the nuclear compartment. A mask of the nuclear space was created from the DAPI image and superimposed on the Texas Red image. Thus, the resulting masked image was separated into 2 distinct regions: the nuclear area (the foreground), which displayed protein contained primarily within the nucleus, and the remaining extranuclear space (the background), which was shown as a black area (Figure 2B). As described below, the foreground was then analyzed by 2 different methods to determine the total nuclear fluorescence for each image, as well as the variability among individual nuclei within a single image.

Analysis of Total Nuclear Localized Protein
To identify overall differences in the nuclear expression of NF-κB, Egr-1, c-Jun, and c-Fos in cells exposed to disturbed laminar shear stress versus those subjected to uniform flow or static conditions, we determined the average fluorescence (gray-scale value from 0 to 4095) for the total foreground area for each masked image (i.e., all nuclei combined). This average fluorescence was then plotted as a function of position in the flow field (see Figures 3B, 4B, 5B, and 6B).

Cellular Heterogeneity of Nuclear Protein Content
Our initial analysis indicated that not all cells within a given image field displayed comparable nuclear expression of a given transcription factor. This suggested that there might be significant variability in cellular responsiveness to biomechanical stimulation, even in cells exposed to the same flow conditions. In order to quantitatively analyze this, we first utilized a computerized algorithm to separate the foreground of each masked image into individual nuclei. Then we determined the average fluorescence for each individual nucleus in images of endothelial cells from 3 different regions: disturbed laminar shear stress (specifically, images in the second and third fields downstream from the bar), uniform laminar shear stress (images in the ninth and tenth fields), and static conditions. The resulting data were displayed in histogram format to reveal the differences in population distributions (see Figure 7). Note that analysis of individual nuclear fluorescence is quite data intensive (70 nuclei per image field, up to 30 image fields per coverslip, 8 shear stressed and 8 static coverslips per experiment). Thus, analysis of the total nuclear area (foreground), as opposed to analysis of individual nuclei, was used most frequently in this report.

NF-κB
Disturbed Laminar Shear Stress Enhances Nuclear Localization of NF-κB (p65)
NF-κB is a rapidly activated transcription factor composed of protein dimers of the Rel/NF-κB family, with the p50/p65

Figure 3. Induction of NF-κB (p65) in the spatially disturbed flow model. HUVEC monolayers were maintained under static conditions or exposed to the disturbed flow model for 30 minutes. After fixation and permeabilization, the monolayers were stained for human NF-κB (p65), digitized images were acquired in the disturbed and uniform flow regions, as well as on static coverslips (A, original magnification ×250), and the amount of nuclear localized NF-κB (p65) protein was determined from those images (B), as described in Methods and illustrated in Figure 2B. Data are presented as mean nuclear fluorescence per image ± SD (n = 5 images for static conditions and 6 images for shear stress). Inverted arrowhead indicates reattachment point. ∗P < 0.01 versus static, ∗∗P < 0.01 versus uniform laminar shear stress. The results illustrated in this figure are representative of 3 independent experiments.
In unstimulated cells, the Rel/NF-\(\kappa\)B dimers are generally sequestered in the cytoplasmic space by binding to members of the I\(\kappa\)B family of inhibitor proteins. On cellular activation, NF-\(\kappa\)B is released from I\(\kappa\)B and translocates to the nucleus where it regulates transcription of multiple target genes that have potential pathophysiologic relevance to cardiovascular disease.\(^{29}\) We chose to investigate the expression characteristics of the p65 subunit in endothelial cells in the disturbed flow model because p65 appears to contain the most transcriptionally active sites. HUVEC monolayers maintained under static (no flow) conditions in a quiescence medium (low serum, minus growth factors) displayed very little p65 protein in their nuclei, as detected by immunofluorescence microscopy (Figure 3A). After exposure of cells to disturbed laminar shear stress for 30 minutes, quantitation of the nuclear p65 content, as described above, revealed significant increases (maximal gray-scale value = 674) compared with cells subjected to uniform laminar shear stress (average grayscale = 363) as well as those maintained under static conditions (average grayscale = 291; Figure 3B). Although nuclear localization of p65 within the uniform flow region was slightly greater than that in static cells, this difference was not statistically significant. The numerical data presented here are representative of at least 3 independent experiments.

Endothelial Cells Within the Disturbed Flow Region Exhibit Greater Population Diversity of Nuclear NF-\(\kappa\)B (p65) Content

Not all cells within a given image field appeared to stain equally for nuclear p65. Quantitative image analysis confirmed this cellular variability and also revealed that the population diversity was greatest in endothelial cells exposed to disturbed laminar shear stress (see Figure 7A). Cells subjected to uniform flow and, to a lesser extent, those maintained under static conditions also exhibited variability in p65 expression, but this diversity was notably less than that seen in the disturbed flow region. Because each experiment was conducted with a HUVEC culture derived from a single umbilical cord, this heterogeneity was presumably not because of genetic variability within the cellular population.

Egr-1

Egr-1 is an immediate-early response gene product which generally has very low expression levels in endothelial cells under unstimulated conditions, but undergoes rapid synthesis and nuclear translocation after the cellular activation that occurs in various response-to-injury settings.\(^{30}\) Consistent with these known molecular mechanisms, HUVEC maintained under static conditions in quiescence medium displayed very little Egr-1 protein (Figure 4A). After exposure to
disturbed laminar shear stress for 30 minutes, nuclear localized Egr-1 protein content was significantly increased (maximal greyscale=826) compared with cells exposed to either uniform flow (average greyscale=561) or static conditions (average greyscale=421; Figure 4B). In addition, cells in the disturbed flow region exhibited the greatest cellular heterogeneity of Egr-1 protein within the nuclear space (see Figure 7B). The numerical data presented here are representative of 2 independent experiments.

c-Jun
In general, activation of c-Jun can occur at 2 levels: phosphorylation of c-Jun/c-Jun and c-Jun/c-Fos protein dimers bound to their cognate (AP-1) DNA binding sites, as well as rapid de novo synthesis of c-Jun protein.31 In the experiments reported here, static (no flow) HUVEC monolayers displayed very high levels of nuclear localized protein (Figure 5), compared with the corresponding basal levels of NF-κB, Egr-1, and c-Fos (see below), despite preconditioning in quiescence medium. Nonetheless, the subsequent upregulation of nuclear localized c-Jun by disturbed laminar shear stress (maximal greyscale=1941) was much greater than that seen for any of the other transcription factors studied. In addition, there was a statistically significant induction of nuclear c-Jun content in cells subjected to uniform laminar shear stress (average greyscale=1253) compared with static cells (average greyscale=823), unlike NF-κB or Egr-1. Static endothelial monolayers also exhibited a remarkably extensive population distribution of nuclear localized c-Jun protein (see Figure 7C), which was further broadened by exposure to uniform and, especially, disturbed laminar shear stress. The numerical data presented here are representative of at least 4 independent experiments.

c-Fos
Like c-Jun, c-Fos may be activated both transcriptionally and post-translationally, the latter via alterations in its phosphorylation state. However, in most settings the primary activation mechanism of c-Fos protein occurs at the level of transcription.31 Not surprisingly, c-Fos protein levels were quite low in static (no flow) HUVEC cultures grown in quiescence medium (average greyscale=397; Figure 6). Nuclear localized c-Fos was induced by disturbed flow (maximal greyscale=1018) to a greater extent than that seen for either NF-κB or Egr-1, and like c-Jun, c-Fos levels were also significantly increased by uniform flow versus static conditions (average greyscale=543 and 397, respectively). The population distribution of nuclear c-Fos was larger in cells in
the disturbed flow region and, to a lesser extent, in the uniform flow region, compared with static cells (see Figure 7D). The numerical data presented here are representative of 2 independent experiments.

Discussion
Because of the elaborate geometry of the branching arterial network in mammals, physiological blood flow is inherently complex and nonuniform. Blood flow perturbations are especially enhanced at anatomical sites such as vascular bifurcations, ostial openings, and curvatures–loci that also have increased predilection for atherosclerotic development in humans and certain experimental animal models. Because the flow patterns in these atherosclerosis-prone regions are particularly complicated, the exact nature of the important biomechanical stimulus–one capable of inducing endothelial alterations associated with lesion development–has been a topic of much interest. Initial fluid mechanical investigations identified atherogenic sites as having relatively low fluid shear stress levels at the endothelial lining of the vascular wall. Accordingly, early in vitro studies focused on the different stimulatory effects of low and high shear stress levels in the regulation of endothelial structure and function. Subsequently, more detailed analyses of in vivo disturbed flow patterns have revealed both that the blood flow at these sites remains laminar throughout the cardiac cycle, and that it is uniquely characterized as having 3 distinct regions: separation of the flow streamlines from the vessel wall, reversal of flow, and reattachment of the forward streamlines to the vessel wall (see Figure 1). This fluid mechanical profile results in extremely large spatial gradients of shear stress at the vascular wall, with the maximum gradient occurring near the point of reattachment.

To analyze more precisely the regulatory effects of disturbed flow on endothelial cell structure and function, we developed an in vitro model within the cone-plate apparatus, which incorporates regions of both disturbed and uniform laminar shear stress (see Figure 1). Previous studies with this experimental system have indicated that long-term exposure (on the order of hours to days) of cultured endothelial cells to spatially disturbed laminar shear stress leads to changes in cell shape as well as enhancement of proliferation and migration near the reattachment point. These studies provided the first evidence that gradients of shear stress, as opposed to absolute shear stress magnitudes, could profoundly influence important cell biological responses in endothelium. Subsequent work by DePaola et al., using a modification of this disturbed flow system within a parallel

Figure 6. Induction of c-Fos in the spatially disturbed flow model. HUVEC monolayers were maintained under static conditions or exposed to the disturbed flow model for 30 minutes, then processed for immunohistochemical analysis. (A) Representative image fields from static, disturbed flow, and uniform flow regions (original magnification ×250); (B) quantitative image analysis (performed as described in Methods). Data are presented as mean nuclear fluorescence per image±SD (n=6 images each for static conditions and shear stress). Inverted arrowhead indicates reattachment point. *P<0.01 versus static, **P<0.01 versus uniform laminar shear stress. The results illustrated in this figure are representative of 2 independent experiments.
Thus, spatial shear stress gradients appear to influence the expression of individual endothelial genes and their protein products.

To broaden the scope of the investigation of this problem, we chose to examine the activation patterns of transcription factors that are known to bind to various SSREs and thus regulate the expression of multiple endothelial genes. However, because of the experimental configuration of the disturbed flow system—i.e., the small size of the disturbed flow region (≤ 2 mm in width)—the application of many standard analysis techniques was precluded (e.g., Northern blot analysis, transfected promoter/reporter genes, flow cytometry). Thus, it was necessary to devise a novel examination technique. The image acquisition and analysis algorithms described in this report enabled detailed investigation of nuclear regulatory events on several levels. First, this technique made feasible the correlation of spatially distributed cellular responses with shear stress variations that occurred over very small dimensions. Second, it was possible to visualize the intracellular location of transcription factors and quantify the amount of protein in specific subcellular compartments. Finally, the image analysis techniques utilized in this report permitted population distribution studies on a cell by cell basis within a given area of defined flow. It should be recognized that the nuclear localization algorithm used in these studies may include some cytoplasmic transcription factor in the measure of nuclear protein content (i.e., cytoplasmic protein located immediately above or below the nucleus).

However, several reports have shown that the distributions of the cytoplasmic space in this region of the cell are fractionally small in comparison with the nuclear space, and it is also well documented that activated NF-κB, Egr-1, c-Jun, and c-Fos each translocate efficiently from the cytoplasm into the nucleus via defined molecular mechanisms.

The data resulting from this novel analysis strategy demonstrate that a nuclear genetic regulatory event can be rapidly activated in endothelial cells subjected to disturbed flow for as little as 30 minutes—well before any significant alterations in cellular or monolayer architecture can occur. Even though NF-κB, Egr-1, c-Jun, and c-Fos are regulated through quite different molecular activation mechanisms, the nuclear localization of each of these transcription factors is selectively enhanced by disturbed laminar shear stress compared with uniform laminar shear stress. These findings have important implications for the potential subsequent regulation of multiple endothelial genes because all 4 transcription factors are known to interact functionally with previously identified SSREs. In addition, not only do endothelial cell populations respond differently to disturbed versus uniform flow, individual endothelial cells also display a significant heterogeneity in their responsiveness to disturbed flow, as measured by changes in their nuclear content of NF-κB, Egr-1, c-Jun, and c-Fos. This heterogeneity is particularly striking given the fact that these experiments were conducted with endothelial populations derived from single human umbilical cords, compared with pooled endothelial cultures obtained from multiple umbilical cord donors of diverse genetic backgrounds. Conceivably, such variability could reflect microscopic variations in the local shear stress environment at the surface of individual endothelial cells. Using atomic force microscopy together with computational fluid dynamics in a uniform flow model, Davies et al. identified significant topographical heterogeneity within an endothelial monolayer and showed that such geometrical variations would lead to large variations in the resultant fluid mechanical profile at the cell surface, even in a nominally uniform flow field. Further investigations aimed at correlating patterns of gene expression with local shear stress gradients at the level of individual cells may shed light on this issue.

Previous studies have indicated that uniform laminar shear stress can regulate the activity of NF-κB, Egr-1, c-Jun, and...
DNA binding by AP-1 (the transcription factor complex reported in these experiments. Exposure of endothelial cells qualitative immunofluorescent staining. However, the quantitative image analyses in the present study indicated only a small increase of nuclear NF-κB content in HUVEC in the uniform flow region of confluent monolayers after 30 minutes of flow, compared with static cells, and this induction was not statistically significant. These differences might reflect differences in the quantitative aspects of the distinct examination techniques used (digitized image analysis versus qualitative immunofluorescent visualization). Also, in the experiments described here, the quiescent medium that was utilized contained low serum levels (5% FBS) and lacked both heparin and growth factors, in contrast to the earlier work. Such experimental details are undoubtedly important in evaluation of uniform flow data regarding Egr-1, c-Jun, and c-Fos as well. In the case of Egr-1, previous work in our laboratory, using both Northern blot analysis and transfection experiments with a luciferase reporter construct, demonstrated significant upregulation by uniform laminar shear stress at the level of steady state message and transcription. Preliminary experiments utilizing the image analysis strategy used here demonstrated that these uniform laminar shear stress-induced nuclear Egr-1 changes were comparable in magnitude with those induced by a maximally effective concentration of phorbol 12-myristate 13-acetate (PMA) (T.N., unpublished data, 1996). In the case of c-Fos, others have demonstrated that nuclear localized protein, as detected by image analysis of fluorescently stained endothelial cells, is significantly increased after a 1-hour exposure to high levels of uniform laminar shear stress (25 dynes/cm²), whereas low levels of shear stress (4 dynes/cm²) resulted in a more modest induction without preferential nuclear localization. However, the method for identifying the nuclear space was not reported in these experiments. Exposure of endothelial cells to uniform laminar shear stress induced a biphasic pattern of DNA binding by AP-1 (the transcription factor complex composed of c-Jun/c-Jun and c-Jun/c-Fos protein dimers), with peak activity at 20 minutes and 2 hours. Indeed, whereas significant differences in nuclear localized protein were detectable in the present studies after a 30-minute exposure to disturbed flow, it should be noted that this time point may not correspond to maximal expression for all 4 transcription factors in this model system.

In addition to spatial shear stress variations because of anatomical factors, in vivo blood flow patterns also have temporal nonuniformities resulting from the cardiac cycle. Disturbed flow in vivo is accentuated by the pulsatile blood flow, which effectively moves the reattachment point forward and backward along the arterial wall with each heart beat, creating time-dependent fluctuations in the spatial shear stress patterns. Therefore, in certain vascular geometries, a given endothelial cell may be exposed repetitively to relatively high, forward flow at one instant, low or zero flow the next instant (at the reattachment point), then reverse flow. In vitro experiments have demonstrated that the temporal profile of an imposed shear stress stimulus can significantly alter endothelial structure and function. In particular, recent experiments by Frangos et al with various ramp, step, and impulse flow models have shown that the kinetics of the flow onset are important factors in the regulation of certain endothelial genes, including the gene encoding c-Fos, even at extremely short time points (seconds). Thus, both the temporal and spatial components of fluid shear stress gradients appear to be important in regulating endothelial cell biology in vivo. The steady disturbed flow model utilized in the current report was designed to isolate the spatial variables from the temporal. However, it should be noted that theoretically, even in this model, there is an instantaneous temporal gradient of shear stress that is generated with the onset of flow. As an additional approach, unsteady disturbed flow patterns that incorporate both temporal and spatial fluctuations in the form of a regular to-and-fro movement of the reattachment point could be created within the cone-and-plate system by utilizing an asymmetric cone. Further studies utilizing this and other model systems will be needed to provide insights regarding the importance of combined temporal and spatial shear stress fluctuations in the regulation of endothelial gene expression.

In conclusion, the data in the current report indicate that spatial shear stress gradients, not merely absolute shear stress magnitudes (ie, high versus low shear), represent variables that may have important roles in gene regulation in vivo in settings such as atherogenic loci. Further studies are needed to elucidate the potential interplay of both temporal and spatial fluctuations in the regulation of endothelial gene expression and thus more precisely define these components of this biomechanical paradigm of endothelial activation. Ultimately, these studies should lead to a more comprehensive understanding of the cellular and molecular biological mechanisms that regulate endothelial phenotype in both atherosclerosis-prone and atherosclerosis-protected hemodynamic environments in vivo.

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