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Gene Expression Profiling of Vascular Endothelial Cells Exposed to Fluid Mechanical Forces: Relevance for Focal Susceptibility to Atherosclerosis

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Gene expression profiling has revealed that cultured vascular endothelial cells (EC) respond to fluid mechanical forces by modulating the mRNA level of a large number of genes. However, differences between the gene arrays and the experimental conditions employed by different researchers make comparison between data sets difficult, and limit the interpretation of the results. Despite these problems, analysis of recent data indicates that the transcriptional response of cultured EC to low-shear disturbed flow conditions similar to those at atherosclerosis-prone areas is distinct from that elicited by atheroprotective high shear laminar flow, providing a molecular basis for the focal nature of atherosclerosis. Many of the genes altered by disturbed flow are involved in key biological processes relevant to atherosclerosis such as inflammation, cell cycle control, apoptosis, thrombosis and oxidative stress. Overall, the gene expression profiling data are consistent with the hypothesis of the hemodynamic etiology of atherosclerotic predilection, *viz* that at predilected areas *in vivo* the presence of low shear, nonlaminar flow is sufficient to induce a gene expression profile that pre-disposes the endothelium to the initiation and development of atherosclerotic lesions.

Keywords Atherosclerosis, Endothelial, Microarray, Shear Stress

FLUID MECHANICAL FORCES

The pulsatile flow of blood through the vasculature produces biomechanical forces that influence the function of blood vessels. These hemodynamic forces include circumferential stress due to the pulsatility of blood flow, hydrostatic pressure, and wall shear stress. Shear stress is defined as the frictional force created by the flowing blood and this force acts almost exclusively on the ECs[1] that line the lumen of the vessel. Within straight sections of blood vessels, the shear stress is essentially laminar, but at curvatures or bifurcations, shear stress becomes turbulent

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[1]*Abbreviations:* BMP4, bone morphogenic protein 4; b-myb, myeloblastosis viral oncogene homolog; BST-1, bone marrow stromal cell antigen-1; CGRP, calcitonin gene related peptide; CDK, cyclin-dependent kinase; COX-2, cyclooxygenase 2; CRF, corticotropin releasing factor; DF, disturbed flow; DKK2, Dickkopf-2; DPMR-3, dihydropyriminidinase related protein-3; EC, endothelial cell; Egr-1, early growth response-1; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; ESM-1, endothelial-specific molecule-1; EST, expression sequence tag; GMCSF, granulocyte-macrophage colony stimulating factor (CSF)/CSF-2; gpIIIa, glycoprotein IIIa; HAECs, human aortic endothelial cells; Hsp70, heat shock protein 70; HUVEcc, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; I-KB, inhibitor of transcription factor NF-κB; LF, laminar flow; LDL, low-density lipoprotein; LFA-1, leukocyte cell adhesion molecule (CD18/ CD11A integrin); LSS, laminar shear stress; LPS, Lipopolysaccharide; MAPK6, mitogen activated protein kinase 6; MAPRE2 (RP-1), microtubule associated protein 1; MCP-1, monocyte chemotactic protein-1; MCSF-1, macrophage colony stimulating factor 1; MEK, MAP kinase kinase; MGSA (NAP-3), melanoma growth stimulatory activity alpha/chemokine CXCL1; MIP-2β, macrophage inflammatory protein 2 beta/chemokine CXCL3; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor kappa B; P55CDC (CDC20), cell division cycle (CDC) homologous to yeast CDC20; PAI-1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PYK, protein-rich tyrosine kinase; RANTES, chemokine CCL5; SMC, smooth muscle cell; SOD-2, superoxide dismutase 2; Sp1, transcription factor Sp1; SREBP, serum response element binding protein; SSRE, shear stress response element; TNF-α, tumor necrosis factor alpha; Tsp-1, thrombospondin-1; TSS, turbulent shear stress; VCAM-1, vascular cell adhesion molecule-1; Wnt13, wingless-type MMTV integration site family member 13.

(nonlaminar). It is well established that fluid mechanical forces regulate the expression of specific genes in ECs, at least in vitro, and are important modulators of vascular EC function.

FLUID MECHANICAL FORCES AND ATHEROSCLEROSIS

The risk of developing premature atherosclerosis is dependent upon both genetic and environmental risk factors. Despite the fact that the well-established risk factors are systemic in nature, atherosclerosis tends to develop at branch points and bifurcations in the arterial circulation in both humans and animals (Asakura and Karino 1990; Gibson et al. 1993; Nakashima et al. 1994). The existence of these so called "predilected sites" points to the role of localized, nonsystemic factors in the susceptibility to atherosclerosis. It has been known for some time that predilected sites are characterized by nonlaminar, pulsatile blood flow with low or oscillating levels of shear stress (Ku et al. 1985; Asakura and Karino 1990). More recently, it was also shown that disturbed flow, characterized by a high Reynolds number (>200), correlated with increased risk of adverse events following coronary angioplasty (Kinlay et al. 2002). In contrast, regions of the arterial circulation that are more resistant to atherosclerosis are exposed to pulsatile LF with high levels of shear stress. These observations have given rise to the hypothesis that along with systemic factors (e.g., hypercholesterolemia), local mechanical forces, in particular shear stress, play an important role in the initiation and progression of atherosclerosis.

IN VITRO MODELS OF FLUID MECHANICAL FORCES

In order to study the effects of fluid mechanical forces upon EC function, a number of in vitro cell culture models have been developed. These models mimic various components of the flow conditions present in living vessels. Steady laminar shear stress at physiologically relevant levels (10 dynes/cm^2) can be generated using a cone-and-plate viscosimeter in which a cone submerged in the medium over the EC monolayer is rotated at a constant velocity (Bussolari et al. 1982). Alternatively, cells can be exposed to steady laminar shear stress using a parallel-plate chamber that consists of two glass or plastic plates separated by a thin gasket that forms a chamber with entrance and exit ports at either end. A monolayer of cultured ECs is placed inside the sealed chamber and medium is passed over the cells at a constant velocity. This creates unidirectional laminar flow at a constant shear stress, the magnitude of which is determined by flow rate, height of the chamber, and the viscosity of the medium (Samet and Lelkes 1999). Using either the cone-and-plate or parallel-plate systems, steady laminar shear stress equivalent to the mean shear stress levels that occur in straight sections of arteries in vivo (10 to 20 dynes/cm^2) can be generated. However, this does not mimic the pulsatility of the blood flow experienced by arterial ECs in vivo. Unidirectional, pulsatile flow at a predictable mean shear stress level can also be generated in a parallel-plate chamber (Helmlinger et al. 1991), but few molecular studies have been performed using these conditions.

A number of different in vitro models have been developed in an attempt to mimic various components of the complex flow conditions present at branch points and bifurcations (predilected areas). By alternating the direction of flow of medium over the EC monolayer in a parallel-plate chamber, the cells are exposed to oscillatory laminar flow in which the mean time averaged shear stress is close to 0 (Chappell et al. 1998). Although this model mimics to some degree the oscillatory nature of the blood flow at predilected areas, the flow is still predominantly laminar (not disturbed) and so differs significantly from conditions in vivo. The parallel-plate chamber has also been used to generate low-shear (e.g., 2 dynes/cm^2), unidirectional laminar flow thus reproducing the low-shear-stress component of the conditions present at predilected areas (Mohan et al. 1997), but again the flow is still laminar and nonpulsatile. In yet another model, a vertical step is placed across the width of a parallel-plate chamber at the in-flow side, which creates a localized area with recirculating eddies and a reattachment area (Chiu et al. 1998; DePaola et al. 1999). The reattachment area experiences high shear stress gradients, one of the characteristics of the flow conditions thought to exist at branch points and bifurcations. However, the flow patterns in this model vary with distance from the step, making it difficult to analyze molecular changes in the cells beyond visualization of, e.g., localized nuclear translocation of specific transcription factors such as NF-κB, Egr-1, c-Jun, and c-Fos (Nagel et al. 1999). By adjusting the angle of the cone in the cone-and-plate apparatus, turbulent flow can be generated in the outer region of the culture plate (Garcia-Cardena et al. 2001). However, the time averaged shear stress under these conditions was 10 dynes/cm^2, which is somewhat higher than the shear stress levels thought to occur at branch points and bifurcations (Ku et al. 1985). A circular shaped flow chamber has been used to generate low shear, nonsteady, pulsatile, nonunidirectional flow (Lelkes and Samet 1991; Christensen et al. 1992; Samet et al. 1993), thus mimicking many of the flow characteristics believed to be present at branch points and bifurcations. A more extensive description of this flow system can be found in "Validation of an In Vitro System of Low-Shear, Nonunidirectional Pulsatile Flow" and in the cited literature.

SENSING AND TRANSDUCING SHEAR STRESS

A single layer of ECs forms a barrier between the blood and the underlying smooth muscle cells of the vessel wall, and as such is the only cell type directly exposed to shear stress. Thus it is likely that the endothelium both senses and responds to the mechanical stimuli exerted by the flowing blood and thus mediates its pro- or antiatherogenic effects. It is technically difficult to measure molecular changes associated with exposure to mechanical forces in situ in living vessels. Therefore model systems have been designed in an attempt to mimic in vitro the fluid shear stresses that occur in vivo. Models in which ECs are exposed to steady LF at magnitudes equivalent to those present in vivo (10 to 30 dynes/cm^2) are relatively simple and consequently are

the most well studied to date. Although our understanding of the exact molecular mechanisms used by ECs to sense and respond to laminar shear stress is not yet complete, it is clear that components of the cytoskeleton, particularly integrins, play an important role. For example, laminar shear stress induced alterations in the interaction of integrins with extracellular matrix proteins, leads to signaling through MEK and the small GTPase RhoA, ultimately resulting in changes in gene expression and phenotypic alterations (Shyy and Chien 2002). The activation of another small GTPase, Rac1, by laminar shear stress was shown to mediate both ICAM-1 up-regulation and cytoskeletal rearrangement (Tzima et al. 2002). However, other pathways involving PYK and ERK1/2 have also been implicated. For more complete information on mechanotransduction by ECs, several excellent reviews are available (Takahashi et al. 1997; Chien et al. 1998; Traub and Berk 1998; Davies 2002; Shyy and Chien 2002; Davies et al. 2003). The cellular signaling pathways that respond to shear stress have also been investigated by first identifying shear stress–responsive genes. Promoter analysis has identified several transcription factor binding sites as laminar shear stress response elements (SSREs). The transcription factor Egr-1, which is induced by shear stress, binds to a G/C-rich sequence that overlaps a Sp1 binding site in the PDGF-A gene promoter and is required for the shear stress inducibility of a reporter gene driven by this promoter (Khachigian et al. 1997). Egr-1 was also shown to be required for induction of the tissue factor gene promoter by shear stress in vitro, and levels of Egr-1 were elevated specifically at occluded sites in the rat aorta that experience increased levels of shear stress (Houston et al. 1999). However, the PDGF-B chain gene promoter appears to utilize a distinct SSRE that is activated by the binding of NF-κB p50–p65 heterodimers that accumulated in the nuclei of cultured vascular ECs exposed to 1 h of high-shear LF (Khachigian et al. 1995). Conversely, the up-regulation of VCAM-1 mRNA expression by steady LF at low shear stress (2 dynes/cm^2) was shown to be dependent on down-regulation of I-κB by low shear and the resulting activation of NF-κB (Mohan et al. 2003). If NF-κB is activated by both high-shear and low-shear LF, and this is an important component of flow activated signaling, then similar sets of genes, including VCAM-1, should be activated by both stimuli. Because this is not the case, the role of NF-κB in the response to shear stress needs to be clarified by further investigations. There are reports that additional transcription factors, including Sp-1 (Korenaga et al. 2001; Yun et al. 2002) and SREBP-1 (Liu et al. 2002), play a role in mediating the effects of laminar shear stress. It seems likely that multiple transcription factors and multiple response elements, rather than a single conserved pathway, transduce the laminar shear stress signal to the nucleus of ECs. Although much progress has been made in understanding the signaling pathways activated by steady laminar shear stress, very little work has been done on nonlaminar flow. Whether different mechanosensing and signaling pathways are activated in response to the nonlaminar disturbed flow present at branch points and curved regions of vessels remains to be determined. Recently, the availability of gene expression profiling techniques has enabled researchers to measure changes in the mRNA levels of large sets of genes in response to fluid mechanical forces. These studies, which are the focus of this review, have begun to reveal the differential response of ECs to high-shear LF and low-shear, nonlaminar disturbed flow.

FLOW-INDUCED CHANGES IN EC GENE EXPRESSION

Techniques of Gene Expression Profiling

Microarray technologies enable the transcriptional response of many thousands of genes to be measured simultaneously. However, this technology is still limited by the often arbitrary selection of genes on the array. Although advances have been made towards placing larger and more complete sets of genes on cDNA arrays, it is still not practical to profile the expression of all the genes in the human genome. In addition, arrays containing larger sets of genes tend to be less sensitive and less informative due to the content of EST sequences and carry an increased risk of false positives and false negatives due to the occurrence of cross-hybridization between closely related sequences. For these reasons, we chose to use the Atlas arrays (BD Biosciences-Clontech) that contain only well-characterized genes and utilize cDNA probes on the array that have been selected and tested to eliminate cross-hybridization. In addition, these arrays utilize a mixture of gene-specific primers to synthesize the radiolabeled cDNA that is hybridized to the array. With this method, the complexity of the probe is reduced, thereby increasing sensitivity and significantly reducing cross-hybridization. Although these arrays contain fewer genes than other commercially available or home-made arrays, by selecting sets of genes specific to the vascular system (e.g., the cardiovascular array) or covering a wide variety of cellular functions (e.g., the Human I general array), we were able to generate informative data sets (Brooks et al. 2002). Alternative gene expression profiling techniques that have been used include a PCR-based approach that potentially allows the analysis of a larger set of genes in a less biased fashion (Wasserman et al. 2002). We also employed subtraction cloning as an unbiased method to enrich for genes that were differentially expressed between cultured ECs exposed to different fluid mechanical stimuli (Brooks et al. 2002). Screening the resultant collection of cDNA clones using low-density arrays proved to be an effective way to identify novel flow-responsive genes.

Gene Expression Profiling of ECs Exposed to Laminar Shear Stress

The effects of steady laminar shear stress upon EC function and gene expression have been extensively studied using various in vitro cell culture systems. When ECs are removed from an artery and maintained in culture, they are no longer exposed to the high-shear LF that they had been exposed to in situ.

When ECs are adapted to grow in cell culture conditions, their phenotype changes dramatically. They change from a nonproliferative phenotype to an actively dividing phenotype, and when grown on plastic, they are no longer exposed to signals from the extracellular matrix. These changes can be ameliorated by growing the ECs in dishes coated with matrix proteins and performing experiments only when they have formed a confluent monolayer and become contact inhibited. However, in the absence of flow/shear stress (so called "static" condition), the ECs are still in a nonphysiological state and therefore the gene expression profile they exhibit should be considered a cell-culture artifact. Nonetheless, most of the available data on the effects of shear stress on EC function and gene expression come from a comparison of ECs maintained under static (no-flow) conditions to ECs exposed to uniform, high-shear LF. These experiments are valuable because they clearly demonstrated that ECs respond to shear stress by alterations in gene expression (Gimbrone et al. 1997; Papadaki and Eskin 1997; Chien et al. 1998).

A number of genes induced by uniform laminar shear stress have antiatherogenic potential, for example, manganese superoxide dismutase (MnSOD) and endothelial nitric oxide synthase. This led to the important concept that high-shear LF induces the expression of atheroprotective genes in the endothelium (Topper et al. 1996). Down-regulation of proatherogenic genes by high-shear LF, for example VCAM-1 (Ando et al. 1994), endothelin-converting enzyme (Masatsugu et al. 1998), and angiotensin-converting enzyme (Rieder et al. 1997), were also reported, supporting the hypothesis that high-shear LF is atheroprotective. Initial studies on high shear–induced gene expression changes looked at a small number of genes that were of particular interest or relevance to vessel wall biology. There are now at least six reports that include microarray-based gene expression profiling of steady laminar shear stress (Chen et al. 2001; Garcia-Cardena et al. 2001; McCormick et al. 2001; Brooks et al. 2002; Dekker et al. 2002; Peters et al. 2002), whereas one used a PCR-based method (Wasserman et al. 2002) and another utilized subtraction cloning (Chen et al. 2003). Differences in the type of ECs used (arterial versus venous), the selection of genes on the microarray, the level of shear stress applied, and the time point analyzed makes comparison of these studies difficult. In addition, because these studies are performed with primary cultures of ECs, the impact of genetic differences upon the response to shear stress cannot be ignored. Although most of these studies have examined EC exposed to shear stress for up to 24 h, it is not known if this is sufficient time to allow the establishment of a stable flow–induced gene expression profile. The development of new in vitro flow systems that allow ECs to be remain viable for up to a week (Dekker et al. 2002) will be valuable tools for monitoring long-term responses to shear stress. Gene expression changes that occur in the first few hours after application of shear stress may provide information on how ECs respond to rapid changes in shear stress (Peters et al. 2002). However, these immediate-early responses are often transient, as in the case of MCP-1 (Shyy et al. 1994), that was initially reported to be up-regulated by laminar shear stress at early time points. The up-regulation of MCP-1, which is a proinflammatory molecule, is not consistent with the proposed atheroprotective function of high-shear LF. However, the more recent microarray data showed that MCP-1 expression was decreased by exposure to 6 h or 24 h of steady laminar shear stress (McCormick et al. 2001; Brooks et al. 2002). Another example of the difference between early and late responses is expression of the E-selectin gene, which was reported to be induced sixfold after 1 h of laminar shear stress (Peters et al. 2002), but was unchanged at 2 h or 24 h (Brooks et al. 2002). Thus the physiological relevance of gene expression changes that occur within the first few hours after the switch from static conditions to high-shear LF is unclear. The fact that some gene expression changes were common between the various microarray analyses of ECs exposed to steady LF for 24 h indicates that both the physiological response to shear stress and the microarray methodology are reasonably reproducible. For example, several of these studies report the up-regulation of heat shock proteins and cytochrome P4501B1, and the down-regulation of endothelin-1, MCP-1, caveolin-1, BMP4, and VCAM-1. However, there are also some striking differences between various data sets. For example, 24 h of steady laminar shear stress down-regulated ICAM-1 and PAI-1 mRNAs in HAECs (Brooks et al. 2002), but up-regulated the mRNA for these genes in HUVECs (Wasserman et al. 2002). Interestingly the extracellular matrix protein S1-5 was down-regulated by 24-h steady LF in both these data sets. Unfortunately neither ICAM-1 or E-selectin were present on the microarray used in the study of Chen and colleagues (Chen et al. 2001). These examples indicate the difficulties encountered when comparing expression profile data generated by different laboratories.

To obtain a true consensus expression profile for high-shear LF would require the establishment of a centralized database that would allow the comparison of gene expression data generated with microarrays containing different sets of genes. A further complication is that many of the shear-induced gene expression changes determined from microarray analysis are of low magnitude (twofold or less) and the validity of these measurements needs to be carefully considered given the inherent variability of the methodology. Even when statistical analysis indicates that a small change is significant, the biological relevance is still difficult to determine and will depend on many factors, including the function of the encoded protein. The biological functions of some of the laminar shear stress–regulated genes identified in these array studies as well as in earlier experiments is consistent with the induction of an anti-inflammatory, antiproliferative, and anticoagulant EC phenotype. However, for many of the shear stress–regulated genes, a clear role in vascular protection has not been suggested.

Steady laminar shear stress is the most commonly used model of the mechanical forces experienced by ECs in atheroprotected areas. However, blood flow in the arterial system in vivo is

pulsatile. Using ECs cultured inside a hollow fiber, it was shown that exposure to pulsatile laminar shear stress for up to 7 days altered the expression of genes involved in the production of antioxidant intermediates and control of cell shape (Desai et al. 2002). An effect of pulsatile LF on the expression of "classical" steady LF–regulated genes (e.g., PAI-1, MCP-1, VCAM-1, COX-2, MnSOD) was not reported. However, it is not clear if this was because these genes were not present on the cDNA array used or because they were actually not regulated by pulsatile LF. Exposure of ECs to pulsatile LF for 7 days altered the expression of only a subset of the genes whose expression was altered after exposure to 24 h of steady LF (Dekker et al. 2002). However, it is not clear from this study if these differences are related to the time of exposure to shear stress or differences between the effects of steady and pulsatile flow. A direct comparison of pulsatile and steady laminar shear stress did reveal differences in the distribution of cytoskeleton components and in the expression of eNOS mRNA and protein (Blackman et al. 2002), suggesting that ECs do respond differently to these two stimuli. In the future, it will be important to determine what effect the addition of pulsatility has on the gene expression profile of ECs exposed to long-term laminar shear stress.

Gene Expression Profiling of ECs Exposed to Nonlaminar Disturbed Flow

Cultured ECs exposed to steady laminar shear stress are representative of ECs at straight sections of arteries in vivo. These studies tell us something about the normal gene expression profile of ECs in atherosclerosis-resistant vessel segments, and about the genes whose expression level is modulated by laminar shear stress. However, if we want to understand the role of flow-regulated gene expression in the development of atherosclerosis at predilected vascular regions, it is essential to determine the expression levels of the same set of genes under both laminar shear stress and flow conditions characteristic of atherosclerosis-prone areas. In theory, the gene expression profile exhibited by ECs at predilected areas may be due simply to the absence of the high-shear LF stimulus. Alternatively, the specific flow conditions at predilected areas may represent distinct biomechanical stimuli that alter the expression of specific genes. If the second possibility is true, then the gene expression profile of ECs exposed to disturbed flow will be different from that of cells under both static and LF conditions. In vitro modeling of the flow conditions at predilected areas is complex, partly because accurate measurements of the in vivo conditions do not exist and also because of the engineering difficulties in generating less well-defined flow parameters. In one model, ECs were exposed to oscillatory flow in a parallel-plate chamber in which the flow is essentially laminar but the net time-averaged shear stress is close to zero (Chappell et al. 1998). This model mimics the nonunidirectional nature of the blood flow at predilected areas but is still very different from the in vivo situation. The expression level of the mRNA for VCAM-1, an important adhesion molecule for monocytes and a hallmark of early events in atherosclerosis, was induced by oscillatory flow when compared to cells under static conditions, but the induction was transient (Chappell et al. 1998). Exposure of ECs to steady LF at a shear stress of 2 dynes/cm^2 mimics to some degree the low shear stress levels at predilected areas, and was also shown to up-regulate VCAM-1 mRNA after 6 h when compared to static cells (Mohan et al. 1997, 1999). However, a more comprehensive analysis of gene expression changes in these models has not been reported. Gene expression profiling using cDNA microarrays was performed after exposure of HUVECs to either steady laminar shear stress (LSS) or turbulent shear stress (TSS), both at a high shear stress level of 10 dynes/cm^2 (Garcia-Cardena et al. 2001). By comparing LSS and TSS to static cells, it was clear that these stimuli regulated distinct but overlapping sets of genes. The finding that at least 30 genes were differentially expressed between TSS- and LSS-exposed cells demonstrated that TSS is a distinct biomechanical stimulus. Although this model mimics the nonlaminar nature of the blood flow at predilected areas, the shear stress level is still higher than is generally believed to occur at such regions.

Validation of an In Vitro System of Low-Shear, Nonunidirectional Pulsatile Flow

In an attempt to more closely approximate the flow conditions at predilected areas, Lelkes and colleagues developed an in vitro system (Fig. 1) that combines low shear stress with nonsteady, pulsatile, nonunidirectional flow (Lelkes and Samet 1991; Christensen et al. 1992). The Reynolds number in this circular chamber was calculated to be approximately 500, and the frequency parameter (Womersley number) was calculated to be 10 (Samet and Lelkes 1993), which defines the average flow in the chamber as essentially laminar. However, the use of a circular flow chamber leads to the generation of complex, nonuniform flow patterns that differ between different locations in the chamber, as can be seen in Figure 1. Cells were placed in regions of the chamber (designated positions 2 and 4 in Fig. 1) that experience recirculating eddies and nonsteady, nonunidirectional flow with a low computed time-averaged shear stress level of <0.01 dynes/cm^2 (herein referred to as disturbed flow, DF). Detailed descriptions of the flow characteristics of this flow system have been reported previously (Samet and Lelkes 1993, 1994, 1999).

As an initial validation of this in vitro model VCAM-1 mRNA and protein levels were measured because elevated expression of this gene appears to be a good in vivo marker of EC activation at predilected vascular regions (Li et al. 1993; O'Brien et al. 1993; Cybulsky et al. 2001). Of particular relevance is the observation that VCAM-1 expression is up-regulated in lesion-prone sites in the arteries of normal mice in the absence of diet-induced atherosclerosis (Nakashima et al. 1998; Iiyama et al. 1999). These reports demonstrate that VCAM-1 up-regulation occurs at predilected vascular areas very early in the process of

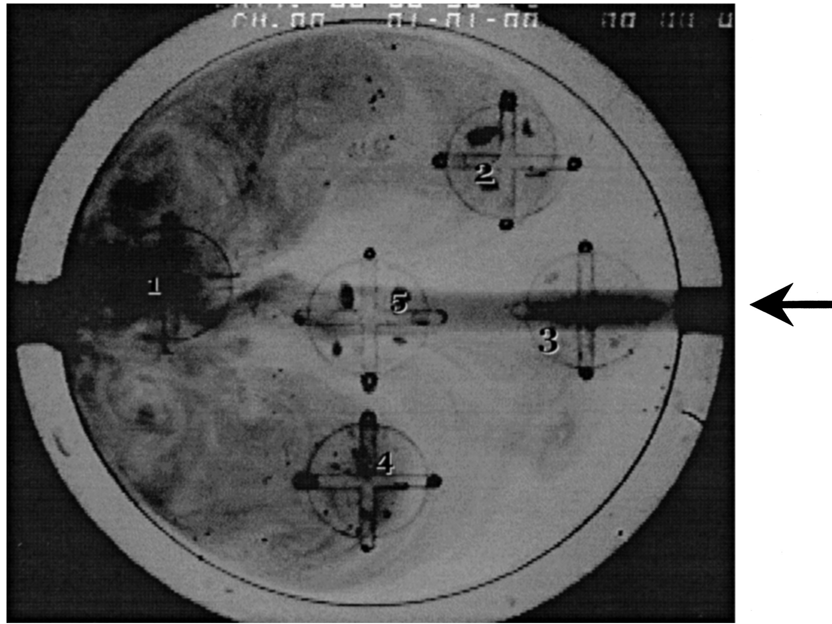

FIG. 1. Visualization of flow in the circular chamber used to generate disturbed flow conditions. Five collagen-coated coverslips covered with a confluent monolayer of HAECs are placed asymmetrically into the chamber at positions designated 1 to 5. The macrograph represents flow visualization analysis, based on dye injection, and demonstrates distinct local flow patterns, including circulating eddies in positions 2 and 4, which were used for analysis of disturbed flow (DF). The shear stress at positions 2 and 4 at the flow rate used (100 mL/min) was calculated at <0.01 dynes/cm^2. The arrow indicates the direction of medium flow through the chamber. Reproduced from Lelkes et al. 2000, with permission.

atherosclerosis in the absence of lipoprotein deposition in the vessel wall, and so is probably a response primarily to local hemodynamic forces. As shown in Figure 2, HAECs exposed to DF in the circular chamber exhibited up-regulation of VCAM-1

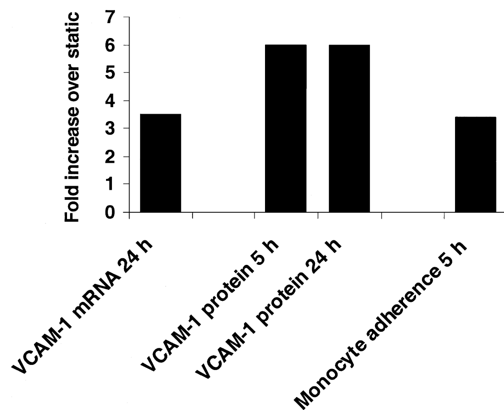

FIG. 2. Up-regulation of VCAM-1 mRNA and protein and increased monocyte adherence in HAEC exposed to DF. Fold changes in VCAM-1 mRNA, protein, or monocyte adherence in HAECs that had been exposed to DF for the length of time indicated as compared to static controls. VCAM-1 mRNA and protein were measured by quantitative RT-PCR and ELISA, respectively. For monocyte adherence assays, DF or static-exposed HAECs were incubated with U937 monocytic cells and adherence was determined by visual counting in three randomly selected fields from duplicate determinations of three independent experiments. Data taken from Brooks et al. 2002, reproduced with permission from the American Physiological Society, and from Lelkes et al. 2000, with permission.

mRNA and protein expression that was sustained for up to 24 h and was associated with a significant increase in monocyte adherence. The fact that VCAM-1 mRNA and protein was up-regulated in a sustained fashion suggests that the mechanical forces in this in vitro model may be similar to those that exist at atherosclerosis-prone sites in vivo (Lelkes et al. 2000).

Comparison of the Gene Expression Profile of ECs Exposed to LF and DF

We have performed transcriptional profiling of HAECs exposed to DF for 6 h or 24 h in the circular chamber, and HAECs under static conditions, using a combination of low-density cDNA arrays (Atlas arrays) and subtraction cloning (Brooks et al. 2002). In addition, we compared the expression profile of DF-exposed cells to that of the same HAECs exposed to steady LF at 13 dynes/cm^2 or tumor necrosis factor alpha (TNF-α). Subtraction cloning was used to enrich for those mRNA species among the entire mRNA population that were more abundant in DF-exposed HAECs than in LF-exposed HAECs at the 24-h time point. Unlike cDNA microarrays, this method is not sequence biased, i.e., not dependent on the arbitrary selection of cDNA sequences present on the array. The combination of subtraction cloning and screening the resultant "DF-enriched" library using low-density arrays proved to be a powerful method for identifying novel DF- and LF-regulated genes (Brooks et al. 2002). DF and LF elicited distinct patterns of gene expression, demonstrating that these are distinct biomechanical stimuli, and supporting the hypothesis that the specific flow conditions at predilected

TABLE 1
Gene expression changes induced by DF and LF at 24 h grouped by expression profile

Expression class	Regulation pattern	Number of genes found by Atlas array	Number of genes found by subtraction cloning	Total	Genes common to both methods
I	DF↑ LF↓	0	15	15	4#
II	DF↑ LF→	4	6	10	0
III	DF→ LF↓	41	11	50	1
IV	DF→ LF↑	13	0	13	0
V	DF↓ LF→	10	0	10	0
VI	DF↓ LF↓	4	0	4	0
VII	DF↓ LF↓↓	3	0	3	0
VIII	DF↑ LF↑	3	0	3	0
IX	DF↑↑ LF↑	3	0	3	0

Note. Regulated genes (at least twofold change) from Atlas arrays and subtraction cloning were grouped according to their expression profile in LF and DF. →, no significant change compared to static; ↑, increased compared to static; ↓, decreased compared to static; LF↓↓, magnitude of down-regulation in LF at least twofold greater than in DF; DF↑↑, magnitude of increase in DF at least twofold greater than the increase in LF.

#VCAM-1, MCP-1, E-selectin, and Tsp-1 fell into class I on arrays generated from subtraction clones, but into class II on Atlas arrays. This is a function of the array technology (Atlas was unable to detect the reduction by LF due to the already low signal intensities for these genes with the static probe).

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areas, and not simply the absence of high shear LF, alters EC gene expression.

The flow-regulated genes could be grouped into nine classes according to their response to exposure to DF and LF for 24 h (Table 1). Genes down-regulated by LF but unchanged by DF was the largest group, but a total of 50 genes were also regulated by DF. The known biological functions of DF- and LF-regulated genes revealed by the combination of arrays and subtraction cloning provided new information about the response of ECs to fluid mechanical stimuli. Genes encoding seven cell adhesion molecules and eight inflammation-related molecules were up-regulated at the 24-h time point in DF as compared to LF. Moreover, the large magnitude of the difference between DF and LF (on the order of 5- to 25-fold for most of these genes) suggests that these changes would have significant proinflammatory effects on the endothelium. Several of the adhesion molecules we identified were not previously known to be flow regulated. For example, sialophorin (CD43), which was down-regulated by steady LF, is a member of the sialomucin family, which act as scaffold molecules to present selectin carbohydrate ligands, thereby increasing the strength of the interactions between leukocytes and ECs (Lasky 1994). Recently, it was demonstrated that CD43 expressed on T cells is a counterreceptor for the macrophage adhesion receptor sialoadhesin (van den Berg et al. 2001). CD43 expressed on ECs may play a similar role in mediating interaction with macrophages. Another gene we identified as flow regulated for the first time was HuMig, whose mRNA was down-regulated by steady LF. HuMig is a chemokine of the CXC subfamily (Farber 1993; Liao et al. 1995) that appears to specifically activate lymphocytes. These data suggest that mechanical forces have the potential to extensively modulate proinflammatory genes.

We also observed the down-regulation of the mRNA level of various cell-cycle regulators in DF compared to LF that was generally consistent with the known antiproliferative effects of LF. Decreased proliferation in ECs exposed to LF is due at least in part to up-regulation of the expression of $p21^{cip1}$, an inhibitor of the cyclin-dependent kinases (CDKs) CDK2 and CDK4, which leads to a cell-cycle block at G1 (Akimoto et al. 2000; Lin et al. 2000). Our cDNA microarray data (Brooks et al. 2002) revealed that the mRNA for another CDK inhibitor, $p57^{kip2}$, was increased in HAECs exposed to LF for 24 h as compared to HAECs under static conditions. High levels of $p57^{kip2}$ would be expected to contribute to a block of cell-cycle progression at the G1 → S transition by binding to and inhibiting the activity of CDK2 and CDK4 (Lee et al. 1995; Matsuoka et al. 1995). The mRNA encoding wee1Hu, which inhibits cell-cycle progression at G2 by reducing the activity of $p34^{CDC2}$ (Tourret and McKeon 1996), was also increased by LF (Brooks et al. 2002). It is possible that the up-regulation of $p21^{cip1}$ and $p57^{kip2}$ as well as wee1Hu via a common LF-induced signaling pathway all play a role in maintaining arterial ECs in a quiescent state under the conditions of high-shear LF. The rate of DNA synthesis was decreased by exposure to LF (Fig. 3), evidence that the observed changes in the expression of $p57^{kip2}$ and wee1Hu were associated with the expected phenotypic change. The expression of $p57^{kip2}$ and wee1Hu was not changed in DF compared to static conditions, suggesting that DF-exposed cells have lost the cell-cycle block conferred by expression of these genes under LF. Indeed, we observed that the rate of DNA synthesis was increased in

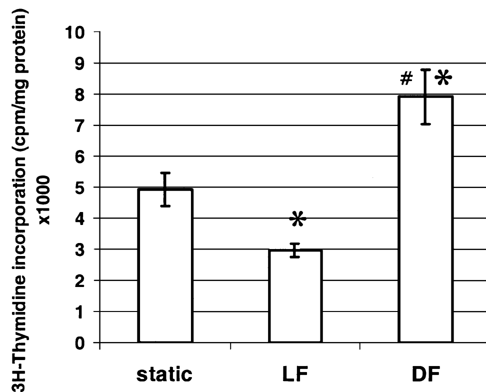

FIG. 3. Effects of DF and LF on DNA synthesis in HAECs. Confluent monolayers of HAECs were exposed for 24 h to DF, high-shear LF, or static conditions. At the end of the experiments, the proliferative status of the cells was assessed by nuclear incorporation of [^{3}H]thymidine. Results are expressed as mean ± *SD* for at least three independent experiments, each performed in duplicate. $*p < .001$ compared to static; $\#p < .001$ compared to LF. Reproduced from Brooks et al. 2002, with permission from the American Physiological Society.

HAECs exposed to DF (Fig. 3). Another gene of interest identified by subtraction cloning as strongly down-regulated by LF (>25-fold) was Dickkopf-2 (Dkk2), a signaling molecule that binds to the LDL receptor–related protein 6 and probably antagonizes Wnt signaling. It has been shown that HUVECs express Wnt-5a and one of the Wnt receptors frizzled-3, and that in brain microvascular endothelial cells, treatment with Wnt activates the Wnt pathway (Wright et al. 1999). An increase in Dkk2 in cells exposed to DF as compared to LF is predicted to block Wnt signaling, but the effect that this has on EC function remains to be determined.

Of particular interest are a group of 15 genes whose mRNA expression was down-regulated by LF and up-regulated by DF (Table 2), most of which were identified only by subtraction cloning. Many of these genes are clearly proinflammatory, such as VCAM-1, E-selectin, and MCP-1. ESM-1 may also be proinflammatory because it was recently shown to bind to the integrin LFA-1 on blood monocytes and lymphocytes and therefore may play a role in modulating the interaction of the endothelium with these inflammatory cells (Bechard et al. 2001). The finding that thrombospondin (Tsp-1), a key inducer of EC apoptosis (Jimenez et al. 2000; Nor et al. 2000), was down-regulated by LF and up-regulated by DF (the net fold up-regulation in DF was more than 40-fold) indicates that mechanical forces may increase the apoptotic rate at predilected areas. These data are consistent with a report that HUVECs exposed to LF had reduced levels of Tsp-1 in the medium (Freyberg et al. 2000). When compared to LF, HUVECs exposed to turbulent flow produced increased levels of Tsp-1 protein in the medium and exhibited an increase in the apoptotic rate that could be blocked by antibodies against Tsp-1 (Freyberg et al. 2001). The gene expression profiling data add to this knowledge by revealing that Tsp-1 expression was increased in ECs exposed to perturbed flow when compared to static conditions. Interestingly,

TABLE 2
Genes that were up-regulated by DF and down-regulated by LF

Accession no.	Gene name	Flow/static DF	Flow/static LF	DF/LF
M30640	E-Selectin	11.3	−12.9	145.5
S71513	MCP-1	3.1	−45.8	144
X89426	ESM-1	6.3	−7.6	47.7
X14787	Tsp-1	3.8	−11.9	42.6
—	Novel clone (Efst1F8)	3.2	−10.5	34
g309181	Pro-collagen	6.8	−3.3	22.7
—	Incyte unique (Efst1G10)	3.4	−6.5	22.4
AB007956	KIAA0487—unknown function	3.2	−6.1	19.8
Af052169	24775 mRNA—K^+ channel	3.3	−6	19.7
X53051	VCAM-1	8.4	−2.5	19.4
X04744	PAI-1	2.2	−5.4	11.1
L76380	CGRP type I receptor	5.2	−1.9	10.1
X02761	Fibronectin	3.8	−2	7.6
CAA63923	MAPRE2 (RP-1)	2.1	−3.6	7.3
D78014	DPMR-3	3	−2.2	6.4

Note. Gene expression differences in HAECs after exposure to DF or LF for 24 h are shown as fold change compared to matched static controls. The data were obtained from genes present on the Atlas arrays and/or genes identified by subtraction cloning. A negative fold change indicates a decrease compared to static. Genes are ranked according to the ratio between DF and LF (last column). Reproduced from Brooks et al. 2002, with permission from the American Physiological Society. See footnote 1 for abbreviations.

the Tsp-1 promoter contains functional and overlapping binding sites for the transcription factors Sp1 and Egr-1 (Shingu and Bornstein 1994), reminiscent of the SSRE identified in the PDGF-A promoter. A role for Tsp-1 in vivo is supported by the finding of Tsp-1 immunostaining in human atherosclerotic plaques (Riessen et al. 1998). An increased rate of EC apoptosis and proliferation at predilected regions that is predicted by both the gene expression data and the in vitro measurements of apoptosis and DNA synthesis would lead to increased EC turnover. This in turn might lead to increased permeability of the endothelium, which in the setting of elevated lipoproteins may contribute to focal deposition of lipids, an important process in lesion formation. The mRNA for the receptor for CGRP, which functions as a potent vasodilator (Brain et al. 1985), was up-regulated by DF and down-regulated by LF. However, the relevance to atherosclerosis of other genes that were up-regulated by DF and down-regulated by LF is less clear, for example procollagen and fibronectin. In addition, the biological function of four of the genes that were up-regulated by DF and down-regulated by LF still remains unknown. The

observation that some genes are regulated only by LF, others only by DF, and some by both is consistent with the concept that these distinct mechanical stimuli activate distinct signaling pathways in ECs. Furthermore, DF may be the local factor initiating a distinct EC gene expression profile and consequent phenotypic changes that contribute to initiation of atherosclerosis at predilected sites.

TNF-α and DF Induce Distinct Gene Expression Profiles in ECs

It has been reported that the up-regulation of VCAM-1 mRNA expression by low-shear LF was associated with activation of NF-κB (Mohan et al. 1997, 1999, 2003). In addition, the protein levels of NF-κB p65 subunit and I-κB were elevated in the regions of the mouse aorta predilected to form atherosclerotic lesions, resulting in higher levels of NF-κB activation in these regions following LPS treatment or a high-fat diet (Hajra et al. 2000). Because transcription of the VCAM-1 gene is inducible by cytokines, acting via the NF-κB response element in the VCAM-1 promoter, it is possible that the up-regulation of VCAM-1 by low shear in vitro and in vivo at predilected areas may be mediated via NF-κB activation. To investigate the role that NF-κB activation might play in the response of HAECs to

TABLE 3

A comparison of gene expression changes induced by DF and TNF-α

Early			Late		
Gene	DF	TNF	Gene	DF	TNF
DF↑ TNF↑			DF↑ TNF↑		
E-selectin	11.6	111.1	E-selectin	11.3	10.6
VCAM-1	7.1	52.8	VCAM-1	8.4	5.4
ICAM-1	6.1	77.1	Potential anti-oncogene	13.6	3.2
MCP-1	6.7	90.8	ESM-1	6.3	6.0
			Jagged 2	4.0	4.0
DF↑ TNF↔			DF↑ TNF↔ or ↓		
Interleukin-6	4.5	1	Cytochrome P450 1B1	62.9	1
Angiopoietin 1	5.0	1	Cytochrome P450 1A1	16.6	1
Insulin-like growth factor BP 1	5.9	1	Incyte unique	7.0	1.4
Heparin-binding EGF-like GF	3.9	1	Hsp70	6.9	−4.2
Homeobox protein Hox-11	6.0	1	CGRP type I receptor	5.2	1.8
			Tsp-1	3.8	1.5
			Collagen 4 alpha 1 subunit	3.6	1
			Wnt13	3.4	1.4
DF↔ TNF↑			DF↔ TNF↑		
			MCP-1	1	137.6
			Sialophorin (CD43, leukosialin)	1	31.5
CRF receptor	1	19.8	BST-1 (CD38)	1	11.6
BST-1 (CD38)	1	10.0	MGSA (NAP-3)	1	10.7
MGSA (NAP-3)	1	14.5	RANTES	1	8.9
Mip-2 β	1	14.1	GMCSF	1	7.4
MCSF-1	1	8.8	Small inducible cytokine B10	1	7.4
SOD-2	1	5.7	Lymphotoxin-beta	1	7.4
			TNF-α	1	7.0
DF↓ TNF↔			DF↓ TNF↔		
PDGF receptor alpha	−16.7	1	P55CDC (CDC20)	−12.8	1
MAPK6 (p97 MAPK, ERK3)	−5.3	1	b-myb	−8.7	1
BMP4	−4.4	1	Cyclin B1	−5.6	1
Neuromedin B receptor	−4.1	1	Caveolin 1	−5.4	1
			Estrogen sulfotransferase	−4.5	1
			Interleukin 17	−4.1	1
			Interferon gamma	−3.6	1

Note. Fold changes in gene expression induced by DF compared to static or by TNF-α compared to vehicle control at two time points. The cutoff for being up- (↑) or down- (↓) regulated was 3.5-fold, and the cutoff for not regulated (↔) was 2-fold or less. Time points were for DF: 2 h (early) and 24 h (late); for TNF: 3 h (early) and 20 h (late). See footnote 1 for abbreviations.

TABLE 4
Quantitative real time RT-PCR analysis of differential gene regulation by DF, LF, and TNF-α

	Fold (flow/static or TNF/control)		
Gene	DF	LF	TNF-α
VCAM-1	3.46	−65.85	79.16
E-selectin	95.25	−1.19	10.64
p57Kip2	1.20	9.98	−1.27
p55CDC	−21.41	−6.07	2.53
Hsp70	7.84	114.12	−4.14
gpIIIa	−1.84	−16.76	−1.17
Jagged 2	2.98	−3.64	−1.63
Tsp-1	3.44	−6.21	1.34

Note. RNA samples from HAECs exposed to DF, LF, or TNF-α for 24 h and the corresponding static or untreated controls were assayed for the genes shown by quantitative-RT-PCR. Quantities of the mRNA for each gene were normalized to the quantity of GAPDH in the same sample that were also measured by quantitative-RT-PCR. For each treatment, the normalized quantity in the treated group was divided by the corresponding control (static for flow experiments and vehicle treated cells for TNF-α) to determine the fold change shown. The fold change for DF is the mean of two independent RNA samples, each of which was pooled from three independent flow experiments. The fold change for LF was determined from a single RNA sample that was pooled from three independent flow experiments, whereas the TNF-α data are from a single treatment. Reproduced from Brooks et al. 2002, with permission from the American Physiological Society. See footnote 1 for abbreviations.

DF, we compared the gene expression profile of HAECs exposed to TNF-α, a potent activator of NF-κB, and DF generated in the circular flow chamber (Brooks et al. 2002). If the transcriptional response of HAECs to DF is due entirely to the activation of NF-κB, then all of the genes up-regulated by DF should also be up-regulated by TNF-α. Although 7 genes, including VCAM-1, were up-regulated by both DF and TNF-α, 37 genes were differentially regulated by these two stimuli (Table 3). We confirmed the differential regulation of several of these genes (Table 4) using quantitative reverse transcriptase–PCR. These results do not rule out the involvement of NF-κB in the response of HAECs to DF, but indicate that additional signaling mechanisms must also be involved.

SUMMARY AND CONCLUSIONS

The recent publication of microarray and other transcript profiling data have revealed that vascular endothelial cells can sense and respond to fluid mechanical forces by up- or down-regulating the mRNA expression level of a significant number of genes. However, the utility of these data sets is limited by differences in the gene arrays used, the type of ECs, and the flow conditions, which makes comparison of different data sets difficult. Ideally, future studies should utilize a common gene array encompassing the most relevant gene set, a single type of EC, and comparative analysis of various flow conditions representative of both the protective and proatherogenic conditions. In addition, it should be appreciated that subtraction cloning or similar techniques that are not biased by the arbitrary selection of genes on a chip can yield valuable gene expression data. Despite these issues, it is clear that the transcriptional response to flow conditions similar to those at atherosclerosis prone areas (disturbed flow) is distinct from that elicited by atheroprotective high-shear LF, and thus provides a molecular basis for the focal nature of atherosclerosis. If the results obtained from the in vitro model systems are representative of the in vivo conditions, these data suggest that the presence of low-shear nonlaminar flow (disturbed flow), together with the absence of high-shear LF, at predilected areas generate a gene expression profile that predisposes the endothelium to the initiation and development of atherosclerotic lesions.

FUTURE DIRECTIONS

The relevance of gene expression data generated from in vitro models depends on how closely these models mimic the in vivo situation. Clearly, pulsatile high-shear LF is a more accurate model of the conditions at atheroprotected regions than steady LF and gene expression profiling of these flow conditions should yield valuable data. Few models of disturbed flow are available, and analysis of gene expression in alternative models that may have different but related flow parameters are needed to support and confirm the existing data. Identification of the promoter elements that mediate the effects of disturbed flow and the transcription factors that interact with these elements should provide valuable information about the signaling pathways activated by this distinct biomechanical force. It is now possible to construct artificial arteries that model atheroprotected and atheroprone areas, seed these with ECs, and maintain them under flow for several days. Gene expression profiling of long-term, stable gene expression in the different regions of these artificial vessels that more accurately model the flow conditions present in vivo should yield valuable data. Recently, it was reported that coculture of ECs with SMCs under static conditions resulted in the up-regulation of the expression of ICAM-1, VCAM-1, and E-selectin that was inhibited by exposure to high-shear LF (Chiu et al. 2003). These results suggest that more accurate modeling of flow responses should also incorporate ECs grown on a layer of SMCs. Finally, it will be important to confirm the gene expression patterns observed in vitro by in vivo analysis. Isolation of RNA from ECs at vascular regions exposed to LF or DF from rodent models is technically challenging but could be amenable to laser capture microdissection followed by linear amplification of cDNA. These techniques have already been applied to cultured ECs (Davies et al. 1999).

Blocking the response of vascular ECs to DF and/or mimicking the response to high-shear LF is an attractive target for therapeutic intervention. There is a strong case for the involvement

of mechanical forces in the initiation of early atherosclerotic lesions. However, early lesion formation is not a practical target for therapeutic intervention because it occurs at an early age and in the absence of symptoms. Although there is no proof that mechanical forces promote the continued development of atherosclerotic lesions, the existing data on gene expression changes in ECs exposed to LF and DF may suggest such a scenario and this needs to be studied in the future. Given that a plethora of genes are regulated by mechanical forces, inhibiting any one of these is unlikely to impact atherogenesis. Targeting components of the flow-activated signaling pathways holds the promise of modulating the expression of multiple genes but also the risk of unwanted side effects. A more complete understanding of the signaling pathways activated by proatherogenic flow conditions as well as by protective laminar shear stress might enable putative therapeutic targets to be identified. Promoter analysis combined with knowledge of the larger sets of genes regulated by mechanical forces gained from expression profiling might enable a clearer picture of the downstream signaling events to emerge.

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