

# Endothelial Dysfunction, Hemodynamic Forces, and Atherogenesis<sup>a</sup>

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**ABSTRACT:** Phenotypic modulation of endothelium to a dysfunctional state contributes to the pathogenesis of cardiovascular diseases such as atherosclerosis. The localization of atherosclerotic lesions to arterial geometries associated with disturbed flow patterns suggests an important role for local hemodynamic forces in atherogenesis. There is increasing evidence that the vascular endothelium, which is directly exposed to various fluid mechanical forces generated by pulsatile blood flow, can discriminate among these stimuli and transduce them into genetic regulatory events. At the level of individual genes, this regulation is accomplished via the binding of certain transcription factors, such as NF $\kappa$ B and Egr-1, to shear-stress response elements (SSREs) that are present in the promoters of biomechanically inducible genes. At the level of multiple genes, distinct patterns of up- and downregulation appear to be elicited by exposure to steady laminar shear stresses versus comparable levels of non-laminar (e.g., turbulent) shear stresses or cytokine stimulation (e.g., IL-1 $\beta$ ). Certain genes upregulated by steady laminar shear stress stimulation (such as eNOS, COX-2, and Mn-SOD) support vasoprotective (i.e., anti-inflammatory, anti-thrombotic, anti-oxidant) functions in the endothelium. We hypothesize that the selective and sustained expression of these and related "atheroprotective genes" in the endothelial lining of lesion-protected areas represents a mechanism whereby hemodynamic forces can influence lesion formation and progression.

## VASCULAR ENDOTHELIUM: A CENTRAL COMPONENT IN THE ATHEROSCLEROTIC DISEASE PROCESS

The involvement of vascular endothelium in disease processes such as atherosclerosis has been recognized since the time of Virchow,<sup>1</sup> but mechanistic insight into the pathobiology of this tissue has developed only recently, largely as a result of the application of modern cellular and molecular biological techniques.<sup>2</sup> We now appreciate

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that the single-cell thick lining of the circulatory system is, in fact, a vital organ whose health is essential to normal vascular physiology and whose dysfunction can be a critical factor in the pathogenesis of vascular disease. It has been our laboratory's working concept that the vascular endothelium is a dynamically mutable interface, whose structural and functional properties are responsive to a variety of stimuli, both local and systemic, and further that its phenotypic modulation to a dysfunctional state can constitute a pathogenic risk factor for vascular diseases. In the arterial wall, certain consequences of endothelial dysfunction are directly related to the pathogenesis of atherosclerosis and its complications.<sup>3</sup> These consequences include altered vascular reactivity and vasospasm; altered intimal permeability to lipoproteins; enhanced mononuclear leukocyte recruitment and intimal accumulation as foam cells; altered vascular cell growth regulation and survival (e.g., decreased endothelial regeneration, increased smooth muscle cell proliferation, enhanced susceptibility to apoptosis); and altered hemostatic/fibrinolytic balances (favoring thrombin generation, and platelet and fibrin deposition). Pathophysiological stimuli of arterial endothelial dysfunction that are especially relevant to atherogenesis include activation by cytokines and bacterial products; infection by bacteria, viruses, and other pathogens; stimulation by advanced glycation end-products (AGEs) generated in diabetes and with aging; chronic exposure to hyperhomocysteinemia and/or hypercholesterolemia; and accumulation of oxidized lipoproteins and their components (e.g., lysophosphatidylcholine) within the vessel wall. In addition to these biochemical stimuli, it is now clear that various biomechanical forces, generated by the pulsatile flow of blood through the branched arterial vasculature, can also influence the structure and function of endothelial cells and even modulate their expression of pathophysiologically relevant genes.<sup>5-7</sup>

The possibility that hemodynamic forces can act directly as pathophysiologic stimuli for endothelial dysfunction provides a conceptual rationale for the long-standing observation that the earliest lesions of atherosclerosis characteristically develop in a non-random pattern, the geometry of which correlates with branchpoints and other regions of altered blood flow.<sup>8-10</sup> In this brief review, we provide an update of ongoing studies in our laboratory focused on the molecular mechanisms involved in the regulation of endothelial gene expression by biomechanical forces, and the new insights they have provided into the pathogenesis of atherosclerosis.

## HEMODYNAMICS AND VESSEL WALL BIOLOGY

The pulsatile flow of blood through the branched tubular array of the arterial vasculature generates various types of hemodynamic forces—wall shear stresses, hydrostatic pressures, and cyclic strains—that can impact vessel wall biology. As the cellular layer in direct contact with blood, the endothelium bears the frictional forces (wall shear stresses) imparted by the flow of this viscous flow. Blood flow patterns can vary in complexity from the relatively uniform (time-averaged) well-developed laminar flow (with corresponding wall shear stresses in the range of 5–15 dynes/cm<sup>2</sup>), that occur in the unbranched portions of medium-sized muscular arteries, to the complex disturbed laminar flow patterns (involving regions of flow separation, recirculation, and reattachment) that result in significant temporal and spatial gradients of wall shear

stress over relatively short distances.<sup>11–14</sup> The latter disturbed laminar flow patterns occur near branch points, bifurcations, and major curves—arterial geometries that are typically associated with the earliest appearance (and subsequent progression) of atherosclerotic lesions. In contrast, the unbranched, tubular portions of arteries that carry uniform laminar flow are relatively protected from atherogenesis (at least in the early stages of lesion formation). For many years, the common wisdom therefore held that low shear areas (e.g., the complex geometries in which the time-averaged fluctuations in wall shear stresses were numerically small, due to forward-reverse flow cycles) were especially atherosclerosis-prone,<sup>15</sup> whereas high shear areas were relatively atherosclerosis-protected (see Refs. 14 and 16 for discussion). Indeed, this nonrandom pattern of atherosclerotic lesion development holds true not only for various experimental models (dietary and/or genetic), across multiple animal species (monkeys, rabbits, pigs, rodents), but also for the natural history of this disease in humans.<sup>9,10,16–18</sup>

A number of *in vivo* observations suggest that hemodynamic forces can alter endothelial structure and function.<sup>19–24</sup> These include the demonstration of increased macromolecular permeability, lipoprotein accumulation, endothelial cell damage and repair, leukocyte adhesion molecule expression, and mononuclear leukocyte recruitment near branch points and bifurcations, as well as the localization of ellipsoidal endothelial cell (and nuclear) shape and axial alignment (in the direction of flow) to laminar flow regions, and the disruption of this orderly pattern in regions of disturbed flow. In addition, experimental alterations of vascular architecture (e.g., surgical coarctation and shunts) have been shown to result in both acute and chronic vessel wall changes that appear to be (at least in part) endothelium dependent. In the presence of hypercholesterolemia, these surgically modified vascular geometries can develop lesions that resemble atherosclerosis. Taken together, these *in vivo* observations are consistent with a direct, or indirect, effect of one or more hemodynamic stimuli on endothelial function/dysfunction in the context of atherogenesis.

Evidence of the direct action of hemodynamic forces on endothelial structure and function has come primarily from *in vitro* studies, in which cultured monolayers of human- and animal-derived vascular endothelial cells have been subjected to defined fluid mechanical stimulation, under well-controlled experimental conditions. Utilizing a modified cone and plate viscometer, in the early 1980s, our group observed that unidirectional steady laminar shear stresses could induce time- and force-dependent cell-shape and alignment changes in cultured endothelial monolayers, which was reversible upon the cessation of flow.<sup>24–26</sup> These shear-induced changes were accompanied by reorganization of actin-containing stress fibers, as well as other cytoskeletal components, thus mimicking the morphology of aortic endothelium *in vivo*. Further studies by our group, and several others, have also documented a variety of changes in the metabolic and synthetic activities of endothelial cells in response to defined biomechanical forces, including the production of prostacyclin, growth factors, coagulation and fibrinolytic components, extracellular matrix components, and vasoactive mediators.<sup>5,27</sup> Some of these more acute, shear-induced changes appear to involve regulation at the level of rate-limiting enzymes or substrate availability (e.g., arachidonic acid release by calcium-sensitive phospholipases and NO production by nitric oxide synthase). However, in the case of delayed responses, in which *de novo* protein synthesis is occurring, transcriptional upregulation of gene expression appears to be stimulated as a direct consequence of exposure to fluid mechanical forces.

### MECHANISMS OF ENDOTHELIAL GENE REGULATION BY BIOMECHANICAL FORCES

*In vitro* studies have demonstrated that the application of physiological levels of laminar shear stress to cultured monolayers of endothelial cells can modulate the expression of a broad spectrum of pathophysiologically relevant genes including: growth factors, such as PDGF-A and PDGF-B; transforming growth factor- $\beta$ ; fibrinolytic factors, such as tPA; and adhesion molecules, such as ICAM-1 and VCAM-1.<sup>6,7</sup> The force-dependencies and kinetic profiles for these various genes show qualitatively different patterns, suggesting that the molecular mechanisms linking an externally applied force to genetic regulatory events in the nucleus are complex. These patterns could reflect a complex interplay of stimuli and responses at several levels, including intracellular second messenger pathways, transcriptional activators and inhibitors, and post-transcriptional effects at the mRNA and/or protein level. To experimentally dissect the molecular mechanisms involved in the biomechanical regulation of endothelial genes, we have utilized a well-characterized cone-plate flow apparatus to expose confluent monolayers of cultured human umbilical vein (HUVEC) or bovine aorta to shear stress (e.g., 5–10 dynes/cm<sup>2</sup>). We have analyzed gene expression by various techniques (e.g., Northern blotting, transfection of shear-responsive reporter gene constructs, nuclear run-on assays, and differential display of expressed transcripts). Initially, we studied individual genes (e.g., PDGF-A, PDGF-B, ICAM-1) as molecular model systems, focusing on the analysis of their promoters and interacting transcriptional factors. More recently, we have begun to analyze the patterns of multiple endothelial genes that are responding in a coordinated fashion to different types of biomechanical stimuli.<sup>28</sup>

Early studies in our group, by Resnick and colleagues,<sup>29</sup> focused on the transcriptional regulation of the human PDGF-B gene, which had previously been shown to be shear-sensitive at the level of steady-state mRNA. Nuclear run-on assays confirmed increased transcriptional activity after one hour of flow exposure, and a reporter gene (consisting of a 1.3 kb fragment of the human PDGF-B promoter, coupled to chloramphenicol acyltransferase), when transfected into BAEC monolayers exposed to laminar shear stress, registered several-fold increased expression compared to “no flow” controls. Through the use of 5′ nested deletional mutations of the PDGF-B promoter, shear responsiveness was localized to a relatively short region situated near the transcriptional start site (position –153 to –101). Oligonucleotide probes spanning this region then were used in gel-shift assays of nuclear extracts from large samples (10<sup>7</sup> cells) of both static and laminar shear stress-stimulated endothelial monolayers. A specific, shear-inducible DNA–nuclear protein complex was consistently observed, which localized to a 12 bp portion within the shear-responsive region. Mutational analysis defined a 6 bp core-binding sequence, GAGACC, which was termed the “shear-stress response element” or SSRE. Nuclear protein–DNA binding events could be demonstrated with probes based on this SSRE as early as 30 minutes after the onset of flow, and thus were consistent with the kinetics and transcriptional activation of the intact endothelial PDGF-B gene, as demonstrated by nuclear run-on analysis. Hybrid promoters consisting of this core-binding sequence (GAGACC) coupled with a non-shear-sensitive reporter gene construct were activated by shear stress, thus demonstrating that the SSRE motif was sufficient to confer shear-responsiveness. Interestingly, computer analysis of gene

sequence databases reveals that there was conservation of the sequence across species (human, murine, feline) within the PDGF-B promoter, suggesting that this mechanism of genetic response to biomechanical stimulation in endothelial cells has been conserved in this gene over many years of evolution.

Further studies have also identified other positive and negative SSREs, in addition to the original SSRE motif identified in the PDGF-B promoter. These include: a TRE (AP-1) site in the human monocyte chemotactic protein-1 (MCP-1) promoter, Egr-1/SP1 binding sites in the PDGF-A promoter, and SP1/Egr-1 binding sites in the proximal promoter of the tissue factor gene. Each appear to mediate shear-induced upregulation of these genes.<sup>30–33</sup> In addition, negative SSREs had been mapped in the promoters of other genes, such as VCAM-1 (an AP-1 consensus sequence in the proximal promoter), which appear to mediate downregulation in response to a shear-stress stimulus.<sup>34</sup>

In parallel with these promoter analyses, considerable attention has also been focused on the influence of biomechanical forces on the expression and activation of various known transcription factors. For example, certain immediate-early response genes, such as *c-fos* and *Egr-1*, whose encoded proteins function as transactivating factors, are directly and rapidly induced by shear-stress stimulation in vascular endothelial cells.<sup>31,36</sup> Other transcriptional factors, such as the NF $\kappa$ B system, show their typical pattern of activation (cytoplasmic-to-nuclear translocation) immediately following the onset of shear-stress stimulus.<sup>35</sup> Studies in our group have demonstrated that NF $\kappa$ B components (p50, p65) can interact directly with the SSRE motif in the human PDGF-B promoter, thus promoting further insight into the transduction mechanisms involved in shear-induced gene expression. Interestingly, these SSREs appear to function in a context-specific manner, such that an element that can mediate a transcriptional response to shear-stress stimulation in the context of one promoter might not do so in the context of another, unrelated, promoter. Thus, these sequence elements appear to function in a manner analogous to promoter elements that mediate transcriptional responses to various humoral stimuli, such as growth factors and cytokines.<sup>28</sup> As discussed below, recent studies of novel genes transcriptionally regulated by biomechanical stimulation in endothelial cells have led to the discovery of new transcription factors.

At the present time, considerable attention is being focused on the fundamental question of the identity, location, and mechanisms of action of endothelial flow-sensitive mechanotransducers. Several distinct molecules (e.g., cell-surface ion channels, various receptor-associated G-proteins, and members of the mitogen-activated and stress-activated protein kinase cascades) are rapidly activated in response to fluid shear stresses applied to the endothelial cell surface.<sup>27,37</sup> In addition, cellular organelles, such as the cytoskeleton, plasma-membrane caveoli, lateral cell-cell junctional proteins, basal focal adhesion complexes, and even the lipid bilayer of the plasma membrane, also appear to be participating in shear-induced endothelial responses.<sup>27,38,39</sup> Finally, various second messengers, including ionized cytosolic calcium, intracellular lipid products of the polyphosphoinositide pathway, and nitric oxide, are generated in the context of flow stimulation. As discussed by Davies,<sup>27</sup> the challenges to understand the interaction of these spatially and temporally disparate components in the dynamic interplay of the endothelial cell's response to biomechanical stimulation are in sorting out where transmission becomes transduction, as well as cause-effect relationships.

### IMPLICATIONS OF COMPLEX FLOW PATTERNS FOR ENDOTHELIAL GENE REGULATION

The endothelial cells lining the branched tubular array of the arterial vasculature are subjected to a broad spectrum of flow patterns depending upon their location. Studies by several laboratories, including our own, using different *in vitro* model systems clearly indicate that endothelial cells can sense differences in the temporal and/or spatial characteristics of flow and translate these biomechanical stimuli into different biological responses. For example, steady laminar flow appears to enhance endothelial survival by suppressing apoptosis,<sup>40,41</sup> whereas turbulent flow can trigger endothelial cell division.<sup>42</sup> Differences in the temporal properties of laminar flow stimulation, generated by instantaneous (impulse) versus gradual (ramp) application of the same final level of shear stress, can elicit very different responses in endothelial gene expression.<sup>39</sup> Similarly, oscillatory versus steady laminar flows elicit marked differences in the pattern of adhesion molecule expression in cultured endothelium.<sup>43</sup> To focus more specifically on the effects of spatial gradients in shear stress on endothelial biology, DePaola and colleagues<sup>44</sup> developed an *in vitro* model system that generates large gradients in shear stress over the relatively small dimensions of a cultured endothelial monolayer, thus mimicking the spatial pattern of flow separation (with reversal), reattachment, and flow recovery associated with arterial bifurcations *in vivo*. Using this *in vitro* spatial disturbed-flow model, dramatic differences in endothelial cell shape, migration, and proliferation have been demonstrated in association with this disturbed flow, as compared with uniform laminar flow.<sup>44,45</sup> In addition, significant differences in endothelial expression of connexin43 at the level of mRNA and protein, and concomitant changes in cell-cell communication via gap junctions, also have been correlated with the presence of shear stress gradients in this model.<sup>46</sup> Recent studies suggest that these *in vitro* observations may indeed have a counterpart *in vivo*.<sup>47</sup> Most recently, Nagel and coworkers<sup>14</sup> have demonstrated that endothelial cell monolayers exhibit significant spatial heterogeneity in the nuclear localization of certain critical transcription factors, including NF $\kappa$ B, Egr-1, c-jun, and c-fos, and that these differences correspond to the local shear stress gradient. Taken together, these studies thus strongly suggest that spatial gradients in wall shear stress, in contrast to absolute shear stress magnitudes (*cf.* Ref. 15) can be important determinants of endothelial responses at the level of gene regulation. Further studies are needed to elucidate the potential interplay of both temporal and spatial fluctuations in the biomechanical regulation of endothelial gene expression and, ultimately, to correlate these stimuli with the endothelial phenotypes actually observed in different *in vivo* biomechanical environments.

### HOW MIGHT FLOW-INDUCED ENDOTHELIAL PHENOTYPIC MODULATION CONTRIBUTE TO ATHEROGENESIS *IN VIVO*?

To more systematically address the question of modulation of endothelial phenotype by biomechanical stimulation, our laboratory has turned to high-throughput molecular biological strategies.<sup>28,48</sup> Specifically, we have used a reverse transcription–polymerase chain reaction–based high-throughput differential display of transcripts to compare the patterns of genes that are upregulated or downregulated in

cultured human endothelial cells in response to physiological levels of steady laminar shear stress, a comparable level of turbulent (nonlaminar) shear stress, and a soluble cytokine stimulus (IL-1 $\beta$ ) at a maximally effective concentration.<sup>48</sup> This approach has revealed distinctive patterns of endothelial gene expression not previously appreciated, including a set of genes that appear to be upregulated in a sustained fashion by steady laminar shear stress, but not by turbulent shear stress. Certain of these differentially regulated transcripts encode known endothelial genes of relevance to atherogenesis, such as eNOS (the endothelial isoform of nitric oxide synthase), COX-2 (the inducible isoform of cyclooxygenase), and Mn-SOD (manganese-dependent superoxide dismutase). These endothelial genes encode enzymes that exert potent anti-thrombotic, anti-adhesive, anti-proliferative, anti-inflammatory, and anti-oxidant effects, both within the endothelial lining and in interacting cells, such as platelets, leukocytes, and vascular smooth muscle. The biological consequences of these steady laminar shear upregulated endothelial genes thus would be predicted to be vasoprotective or anti-atherogenic.<sup>2,7,8</sup>

Given the well-established observation that uniform laminar shear stresses are characteristically associated with atherosclerotic lesion-protected arterial geometries *in vivo*, these molecular biological observations have led us to hypothesize that this type of biomechanical stimulation acts to chronically upregulate the expression of a subset of "athero-protective genes" in endothelial cells, which then act locally in the lesion-protected areas to offset the effects of systemic risk factors, such as hypercholesterolemia, hyperhomocysteinemia, hyperglycemia (diabetes), and hypertension. The coordinated and selective upregulation of athero-protective genes by uniform laminar shear stress is thus a possible mechanistic link between the local hemodynamic milieu, endothelial gene expression, and early events in atherogenesis. This working hypothesis, of course, does not exclude the potential direct action of complex disturbed laminar flows, such as occur in lesion-prone arterial geometries, as stimuli for the expression of pro-atherogenic genes (e.g., adhesion molecules, growth factors, cytokines).<sup>2,7,8</sup>

Critical testing of this "athero-protective gene hypothesis" will depend upon refinement of both *in vitro* and *in vivo* fluid mechanical models and a validation of candidate athero-protective genes in the setting of human vascular pathobiology. The development of reliable methods for linear amplification of transcripts from small numbers of cells and their analysis by cDNA micro-arrays or analogous genome-scale technologies should hold much promise in this regard. Application of these comprehensive and relatively unbiased methods of molecular analysis to endothelial cells subjected to experimentally defined flow conditions will add significantly to our understanding of the dynamic range of biomechanically induced phenotypic modulation. Ultimately, the extension of this method of analysis to endothelial phenotype in the natural disease context should provide valuable new insights into the links between endothelial dysfunction, hemodynamic forces, and atherogenesis.

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### Questions and Answers

G.K. HANSSON (*Karolinska Hospital, Stockholm, Sweden*): This kind of experimental design, an unbiased analysis of gene expression that you showed, should permit an analysis of how these different factors interact. For instance, how will a certain biomechanical milieu affect the response to a biochemical stimulus, let's say in a certain disturbed flow, for example.

GIMBRONE: We have spent almost a year now retooling to do this type of high through-put analysis of multiple, interacting stimuli. Differential display lends itself very nicely initially to acquiring a lot of data, but then you spend a lot of time, since you don't know the sequence of each band in the differential display gels. We are now moving to cDNA array transcriptional profiling, where each dot of the 5,000 plus on a given array will have information content. Differential display is great for gene discovery, but it is not as useful for describing the behavior of multiple known genes. Transcriptional profiling is the method of choice.

P. GANZ (*Brigham and Women's Hospital, Boston, MA, USA*): You showed a number of genes that were regulated by either laminar or turbulent flow, but, in your early slides, you also showed a time course for laminar shear showing that some genes go up for a few hours and then they come down. Therefore, I wonder, for those of us who are older than four or six hours, in whom something may have gone up and already returned to baseline, what is the *in vivo* translation of these experiments? Also, specifically, for COX-2, have you shown this protein in vascular endothelium *in vivo*?

GIMBRONE: The immunostaining that I showed for mouse aorta actually was COX-2 protein. The endothelium is uniformly stained except around the ostia. Regarding the temporal patterns of gene expression, you are asking a very important question. All these *in vitro* models are a change from something to something else. The static culture condition is very non-physiologic; it is not the way an endothelial cell normally lives. It is not until you precondition under shear and then change from that more physiologically relevant baseline that you may actually be able to see the interplay of multiple components. So the issue of the "set-point" of the cell is very important. For MnSOD, COX-2, and eNOS, as long as you apply laminar shear, they remain upregulated, they do not downregulate. That also appears to be true *in vivo*.

GANZ: Are there differences in vascular beds not explained simply by differences in laminar flow?

GIMBRONE: Yes, almost certainly. But practically, we are often limited by the availability of good reagents (e.g., antibodies) to demonstrate these differences *in vivo*. You saw the nice work earlier with antibodies to human receptors for VEGF and how revealing that was. In our own studies with BSC-2, SMAD-6, and SMAD-7, and

now with the mouse and human data on COX-2, we are beginning to get some insight into vascular bed differences.

P. CARMELIET (*Flanders Interuniversity, Institute of Biotechnology, Leuven, Belgium*): In your list of target genes, I am missing some receptors as well as some angiogenic factors, or receptors for angiogenic factors. Nevertheless, embryonic vascular development is highly influenced by flow. Do you have any insights?

GIMBRONE: I agree with your point. There will be more data forthcoming.

CARMELIET: Is there anything known on the role of turbulence or disturbed laminar flow in terms of tumor angiogenesis?

GIMBRONE: I have been instructed by my fluid-mechanical engineering colleagues that the definition of turbulence is very precise; likewise the definition of disturbed laminar flow is very precise. The size of the vessel that one can model and actually measure flow is a practical limitation. In the mouse aorta, we do not know the near-wall environment, in terms of laminar versus disturbed flow, because it is extraordinarily hard to measure or model flows in that tiny structure, and I am talking about the aorta, let alone the capillary. In general, the wall shears are very low by the time you get down to the capillary level. I do not think turbulence can exist in the microvascular bed.

A.R. TALL (*Columbia University, New York, NY, USA*): The one thing that is not apparently explained by your findings today is how the cytoskeleton might change, mediating the change in the shape of the cell. One expects that it may also be involved in sensing changes in flow. Do you have any insights into how this is mediated?

GIMBRONE: Peter Davies, at the University of Pennsylvania, has done elegant studies on the role of the cytoskeleton as a potential sensing organ for biomechanical deformation and I refer you to his recent work. Years ago, when he was in our group, we also did some measurements of ionized calcium transients that are evoked by changes in shear in the endothelial cell. In fact, shear can be a very important short-term regulator of ionized calcium flux, MAP kinase pathways, and integrin-dependent adhesion to the extracellular matrix—all of which may participate in flow sensing.