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Role of Shear Stress Direction in Endothelial Mechanotransduction

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Abstract: Fluid shear stress due to blood flow can modulate functions of endothelial cells (ECs) in blood vessels by activating mechano-sensors, signaling pathways, and gene and protein expressions. Laminar shear stress with a definite forward direction causes transient activations of many genes that are atherogenic, followed by their down-regulation; laminar shear stress also up-regulates genes that inhibit EC growth. In contrast, disturbed flow patterns with little forward direction cause sustained activations of these atherogenic genes and enhancements of EC mitosis and apoptosis. In straight parts of the arterial tree, laminar shear stress with a definite forward direction has anti-atherogenic effects. At branch points, the complex flow patterns with little net direction are atherogenic. Thus, the direction of shear stress has important physiological and pathophysiological effects on vascular ECs.

Keyword: Endothelial cells, Gene expression, Mechanotransduction, Proliferation, Shear stress, Signal transduction.

1 Introduction

Endothelial cells (ECs) play important roles in regulating vascular function through their migration, proliferation, and remodeling; the production, secretion, and metabolism of biochemical substances; and the modulation of vascular smooth muscle cell (SMC) contractility, as well as serving as a permeability barrier. ECs respond to not only chemical ligands (e.g., hormones and growth factors), but also mechanical factors such as shear stress in regulating vascular functions under physiological and pathophysiological conditions.

Atherosclerosis causes a narrowing of artery lumen to reduce blood flow and induce the consequent clinical conditions. The two major elements in atherogenesis are low density lipoprotein (LDL) and monocytes. Monocytes are transformed in artery wall into macrophages, which ingest oxidized LDL to form foam cells (Fig. 1). Atheromatous plaques consist of foam cells, SMCs, and extracellular matrix (ECM) that accumulate in the subendothelial intima [Steinberg 1995].

2 Mechano-sensing and Signal Transduction in Response to Shear Stress

Shear stress acts on ECs to activate many types of mechano-sensors to trigger a phosphorylation cascade of signaling molecules, e.g., the mitogen activated protein kinases (MAPKs, which include JNK, ERK and p38) in the Ras signaling pathway. Such mechanotansduction leads to the modulation of expression of chemoattractant genes such as monocyte chemotactic protein-1 (MCP-1), genes concerned with cell proliferation or growth arrest, and many other genes. The changes in gene and protein expressions vary with the mode of shear flow to differenentially regulate the functional behavior of ECs.

2.1 Mechano-sensors for Shear Stress

Experiments performed in our laboratory have demonstrated the participation of a receptor tyrosine kinase and the integrins in the EC sensing of mechanical stimulations and the consequent activation of cellular signaling pathways. In addition, the cell membrane lipid bilayer can also serve as mechanosensors.

2.1.1 Receptor Tyrosine Kinase (RTK)

RTKs are membrane receptors that are activated when become tyrosine phosphorylated upon ap-

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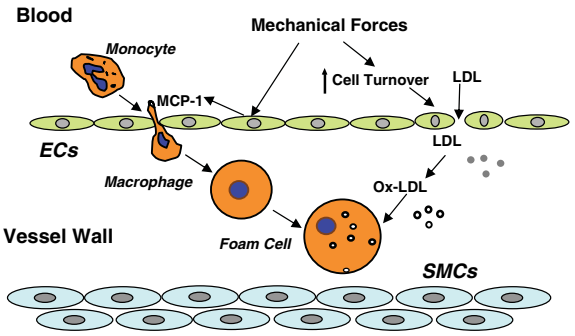

Figure 1: Schematic drawing to show the role of mechanical forces on EC in inducing atherogenesis. The effects of mechanical forces include (a) enhancing cell turnover to increase LDL permeability and (b) causing secretion of MCP-1 to increase monocyte entry across the EC layer. In the subendothelial intima, monocytes are transformed into macrophages, which ingest the oxidized LDL (ox-LDL) to form foam cells. The foam cells and the SMCs that migrate into the neointima are the main cellular elements in the atheroma.

propriate stimulation. The vascular endothelial growth factor (VEGF) receptor Flk-1 is an RTK that responds to its chemical ligand VEGF. Shear stress causes the same responses in ECs as elicited by VEGF, viz., oligomerization and phosphorylation of Flk-1, and its binding to Shc and other adaptor molecules, leading to the activation of Ras and MAPKs [Chen et al. 1999].

Integrins are transmembrane receptors that link the ECM proteins with the intracellular cytoskeletal proteins and focal adhesion proteins to provide inside-out and outside-in signaling between the cell and its ECM [Schwartz 2001]. $\alpha_v\beta_3$ is a major integrin in vascular ECs that interacts specifically with the ECM proteins vitronectin and fibronectin. In response to shear stress, $\alpha_v\beta_3$ associates with the adaptor molecules Shc, Grb2, and Sos to activate the Ras-MAPK pathway; this shear-activation of $\alpha_v\beta_3$ requires vitronectin or fibronectin in the substratum [Jalali et al. 2001]. Laminin is the cognate ECM protein for $\alpha_6\beta_1$ integrin, and shear-activation of EC $\alpha_6\beta_1$ requires laminin in the substratum.

2.1.2 *Interplay between Flk-1 and Integrins in Mechanosignaling*

Wang et al. [2002] conducted experiments to assess the effects of blocking of one of these two membrane proteins on the shea stress-induced activation of the other. Integrin-blocking antibodies such as LM609 abolish the shear-activation of Flk-1, suggesting that integrin is upstream to Flk-1 in its shear-activation. In contrast, the Flk-1 blocker SU-1498 does not affect the shear-activation of $\alpha_v\beta_3$ integrin, suggesting that Flk-1 is not upstream to integrins. The actin-disrupting agent cytochalasin D also abolishes the shear-activation of Flk-1, indicating that the shear-activation of Flk-1 requires the actin cytoskeleton.

2.1.3 *Role of Membrane Lipid Fluidity in Mechano-sensing*

After the application of a shear stress of 10 dyn/cm^2 as a step function, the diffusion coefficient (D) of DiI in the lipid bilayer increases upstream to the nucleus and decreases downstream in less than 10 sec, with both changes disappearing rapidly [Butler et al. 2001]. The upstream D shows a secondary, larger increase, which reaches a peak at 7 min and decreases thereafter under continued shearing. On the downstream side, D shows little secondary changes throughout the 10-min shearing. When the 10 dyn/cm^2 shear stress is achieved with a ramp-up rate of 20 dyn/cm^2 per min [Butler et al. 2002], however, D decreases within 5 sec both upstream and downstream. ERK and JNK are activated by a step-shear of 10 dyn/cm^2, but not by a ramping shear stress (20 dyn/cm^2/min) to the same level. The results indicate that the lipid bilayer of the EC membrane can sense the temporal features of the applied shear stress with spatial discrimination and that this shear-induced membrane perturbations can be transduced to activate MAPKs.

2.1.4 *Summary on Mechanosensing*

Flk-1, integrins and membrane lipids are involved in mechano-sensing. Many other membrane elements can also sense mechanical forces to initiate intracellular signaling. including G-protein coupled receptors, ion channels, and intercellular

junction proteins, as well as membrane glycocalyx (Fig. 2) [Chien 2007].

2.2 Effects of Shear Stress on MAPK Pathway and MCP-1 Gene Expression

The activation of the mechano-sensors and adaptor molecules leads to the modulation of signaling pathways and regulation of gene expression. These adaptor molecules are located near the membrane sensors and in focal adhesions, where they are in close association with proteins such as c-Src and focal adhesion kinase (FAK).

The Ras-MAPK pathway is involved in many cellular functions, including proliferation and programmed cell death (apoptosis). Ras is a common upstream molecule that activates the MAPK signaling pathways with a cascade of phosphorylation of serine-threonine protein kinases, with ERK, JNK and p38 being key downstream molecules. Shear stress causes the sequential activation of Ras and JNK/ERK [Li et al. 1996] to activate the transcription factor AP-1 (composed of c-Jun and/or c-Fos), which activates the TPA responsive element (TRE) to induce the expression of genes such as MCP-1 [Shyy et al. 1995] (Fig. 2). In response to a sustained laminar shear stress, Ras and its downstream molecules undergo a very transient up-regulation followed by a down-regulation.

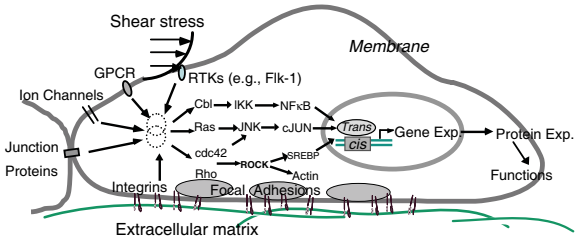

Figure 2: Diagram showing multiple mechanosensors and signaling pathways that may play a role in mechanotransduction in ECs. Potential sensors shown in this figure include Flk-1, G protein coupled receptor, ion channels, junction proteins, and integrins, as well as the cell membrane and glycocalyx. Shear-stimulation of sensors activates the adapter molecules (two dashed circles) and a myriad of signaling pathways (only a few of them are shown here) to modulate gene and protein expressions and cellular functions. Mechanotransduction also leads to cell remodeling through the re-organization of cytoskeleton, including actin. The cytoskeletal elements also modulate signaling pathways. [Modified from Chien 2007].

3 Effect of Shear Stress on EC Proliferation

3.1 Effects of Laminar Shear Stress on EC Proliferation

3.1.1 DNA Microarray Studies on Shear Stress Modulation of Gene Expression

By using the DNA microarray technology, Zhao et al. [2002] have studied the effects of 1-24 hr of laminar shear stress (12 dyn/cm^2) on the expression of a number of genes in cultured human aortic endothelial cells (HAECs). After the relatively long period (24 hr) of shearing, several genes related to EC inflammation and proliferation are down-regulated, suggesting that 24-hr shearing is anti-atherogenic by keeping ECs in a relatively non-inflammatory and non-proliferative state. Genes involved in EC survival and angiogenesis (e.g., Tie2 and Flk-1) and vascular remodeling (matrix metalloproteinase 1) are up-regulated by the 24-hr laminar shear stress. Such beneficial effects of sustained shearing with a definitive direction on EC gene expression can be relevant for the native ECs in the straight part of the aorta *in vivo*.

3.1.2 Roles of p53, p21, GADD45, and Rb Dephosphorylation in the Decrease in Cell Proliferation in Response to Long-term Shearing

Lin et al. [2000] have studied the molecular mechanism underlying the inhibitory effects of laminar shear stress on EC growth. Application of laminar shear stress to BAECs for 24 hr leads to increases in the tumor suppressor protein p53 and the growth arrest proteins GADD45 (growth arrest and DNA damage inducible protein 45) and p21, and a decrease in phosphorylation of the retinoblastoma gene product (Rb). This sequence

of events can lead to the cell cycle arrest found by flow cytometry and hence can provide an atheroprotective function in the straight part of the arterial tree.

3.2 *Effects of Disturbed Flow on EC Proliferation*

We have assessed the effects of disturbed flow on EC proliferation in cultured ECs in a step flow channel, in which flow is disturbed in the short segment after the channel entrance, with flow separation and re-attachment (Fig. 3A). Under static condition, BrdU incorporation is low and randomly distributed throughout the channel. After 24 hr of shear flow at 12 dyn/cm^2, BrdU incorporation is markedly enhanced in the re-attachment area, where flow does not have a clear forward direction, but is much lower in the downstream laminar flow region (Fig. 3B) [Chien 2003]. The same distribution pattern is seen for the expression of proliferative genes and the activation of signaling molecules for proliferation such as ERK. The ERK inhibitor PD98059 can block the increase in BrdU incorporation in the disturbed flow region (Fig. 3C). These results indicate that the disturbed flow pattern in the re-attachment area (seen at artery branch points) stimulates cell proliferation, probably via ERK signaling.

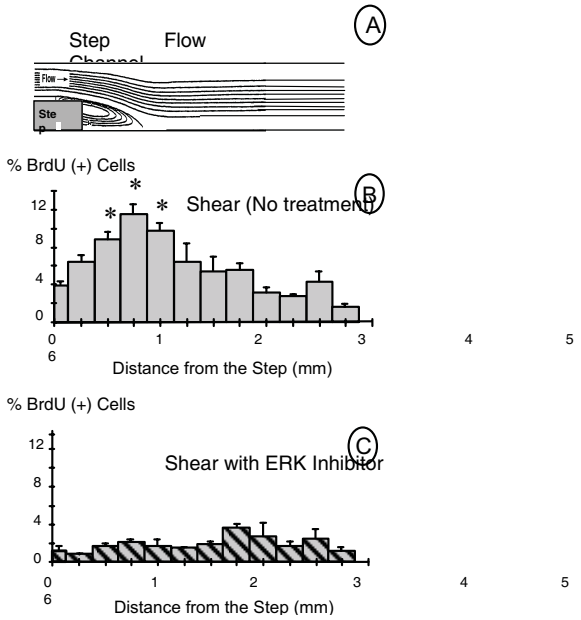

Figure 3: Disturbed flow increases EC proliferation. The step-flow chamber (A) used to ompare the effects of disturbed and laminar flows on EC proliferation. EC proliferation rate (as estimated from BrdU uptake) is elevated in regions of disturbed flow, but not in regions of laminar flow (B). Inhibition of ERK with PD98059 blocked the proliferation effect of disturbed flow (C), indicating that the increase in EC proliferation induced by disturbed flow is mediated by ERK signaling.

4 Effects of Laminar and Disturbed Flows on EC Lipid Metabolism

The activation of sterol regulatory element binding protein 1 (SREBP1) in response to sterol depletion leads to increases in the expressions of genes encoding LDL receptor, cholesterol synthase, and fatty acid synthase, thus restoring the intracellular sterol level. Laminar shear stress (12 dyn/cm^2) causes a transient activation of SREBP1 and the translocation of its transcription factor domain into the nucleus [Liu et al. 2002]. Blockade of β_1 integrin by mAb inhibits the shear-activation of SREBP1, suggesting that integrin plays a critical role in the modulation of EC lipid metabolism in response to shear stress. The shear-activation of SREBP is blocked by cytochalasin D, indicating the importance of the actin cytoskeleton in this effect. Step flow channel studies show that, in contrast to the transient activation of SREBP1 in ECs under laminar flow, disturbed flow causes a sustained activation. As a result, disturbed flow would lead to a sustained increase in the SRE-mediated transcriptional activation of EC genes that promote lipid accumulation.

5 Effects of Laminar and Disturbed Flows on EC Turnover and Macromolecular Permeability In Vivo

The branch points and curved regions of the arterial tree, where blood flow is unsteady and the shear stress shows marked spatial and temporal variations, are the areas prone to atherogenesis. Our experimental studies have provided evidence that EC mitosis [Chien et al. 1988, Lin et al. 1989] and death [Lin et al. 1990] are associ-

ated with the leakage of macromolecules such as LDL and albumin on individual cell basis. Studies performed in several laboratories, including our own [Chuang et al. 1990], have shown that these events of accelerated EC turnover occur primarily in areas with disturbed blood flow such as arterial branch points.

In the rabbit thoracic aorta, the luminal surface has been examined by light microscopy to determine cell and nuclear orientation from the nuclear shape index [S.I. = 4π (Area) / (Perimeter)^2]; a low S.I. indicates elongation whereas a high S.I. reflects a more spherical shape [Chien 2003]. At intercostal orifices, EC nuclei on the upstream side are orientated toward the orifices (Fig. 4) with low S.I. (elongated), but on lateral sides they are oriented diagonally with higher S.I. The nuclear orientation suggests that flow pattern is rather complex even in the straight portion of aorta and that significant disturbance of the flow pattern occurs in small branches, similar to that reported for large branches [Karino 1986].

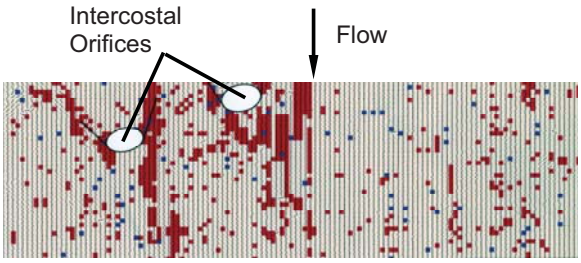

Figure 4: Distribution of leaky spots and mitosis around intercostals orifices, En face preparation of thoracic aorta opened up in the midline of a cylindrical segment. Mitotic cells (blue crosses) and EBA leaky spots (red) are distributed primarily laterally and distal to the orifices. The EBA leaky spots have a V-shaped distribution lateral and distal to the orifices.

In the thoracic aorta, the average frequency of EC mitosis is 0.12% and that of Evans Blue albumin (EBA) leaky spots is 0.40%. While there are very few mitotic cells, 98.8% of them show EBA leakage, and about 1/3 of all leaky spots are associated with mitotic cells. A mitotic cell is often found in the center of the leaky spot, where EBA fluorescence is at its maximum intensity. Mitotic cells and EBA leaky spots are distributed primarily around the orifices of intercostal arteries, i.e., regions with secondary flows or in transitional zones, with the number decreasing laterally away from the orifices (Fig. 4). These distributions of mitosis and EBA-leaky spots are similar to the patterns of lipid accumulation in cholesterol-fed rabbits [Schwenke and Carew, 1989] and of human atherosclerotic lesions [Taxon 1995]. Findings from our and other laboratories provide evidence in support of the roles of disturbed flow in causing atherogenesis [Chien 2007]. The sequence of events that contribute to the focal nature of atherosclerosis involves local hemodynamic factors (complex flow pattern)g, EC turnover gg(mitosis and death), local enhancement of LDL permeability, monocyte entry, and focal lipid accumulation.

As mentioned above, sustained laminar shear flow with a large net forward component down-regulates the MCP-1 gene expression, and this is seen in the straight part of the thoracic aorta. This down-regulation of MCP-1 does not occur in the re-attachment region in vessel branches; in these regions with a lack of sustained laminar shear the MCP-1 expression is higher [Chien 2003]. Mapping of monocyte distribution in the aorta also shows that there is a preferential localization at branch points [Malinauskas et al. 1995]. These results can be interpreted to indicate that the complex flow pattern near the branch points leads to up-regulation of MCP-1 expression and attraction of monocytes, in addition to the lipid accumulation due to increased permeability and sustained up-regulation of SREBP [Liu et al. 2002], thus placing these regions at multiple risks for atherogenesis. In contrast, the sustained shearing in laminar flow regions induces EC growth arrest and down-regulates expressions of MCP-1 and SREBP, thus providing athero-protective actions (Table I).

Table I. Comparison of Flow Patterns, Cellular Events and Atherogenecity between Straight Part of the Aorta and its Branch Points

Many of the effects of disturbed flows can be reproduced by subjecting ECs to oscillatory flow

Table 1: Comparison of Flow Patterns, Cellular Events and Atherogenecity between Straight Part of the Aorta and its Branch Points

	Straight Part	Branch Points
Flow Pattern	Laminar	Disturbed
Net Forward Flow	Large	Small
EC turnover & LDL Permeability	Low	High
Monocyte Adhesion	Low	High
Effect on Atherogenesis	Atheroprotective	Atherogenic

(e.g., 0.5 ± 4 dyncm^2) with a sinsusoidal frequency of 1 Hz, but very little forward direction. In contrast, pulsatile flow with a significant forward direction (e.g., 12 ± 4 dyncm^2) at the same sinsusoidal frequency of 1 Hz, is similar to steady laminar flow in causing growth arrest and other anti-atherogenic effects. Comparison of results on pulsatile and oscillatory shearing [Wang et al. 2006] indicates the importance of the forward component of flow in determining the functional outcome.

6 In Vivo Relevance of Mechanotransduction in Response to Shear Stresses

The endothelium of straight, unbranched vessels is subjected to pulsatile shear stress with a forward direction, and the directional mechanical stimuli cause the ECs in these regions to be elongated and the stress fibers to be oriented such that their main axes lie parallel to the direction of blood flow. The ECs in these straight vessels have a slow turnover rate and low macromolecular permeability, and the signaling processes lead to anti-atherogenic and anti-inflammatory gene and protein expressions, thus favoring the maintenance of vascular homeostasis. In contrast, the geometry at bifurcations in the arterial tree causes complex fluid flow patterns with little forward component [Giddens et al. 1993]. The lack of a definite direction for shear flow in these regions is accompanied by the absence of preferential alignment of stress fibers or orientation of the ECs. The lack of adaptive remodeling of the endothelium at arterial bifurcations is associated with a relatively high permeability to macromolecules and predispositions to inflammation, apoptosis and the development of atherosclerotic lesions [Chien 2003]. Thus, the differences in the direction of shear stress acting on the endothelium may play a critical role in the preferential localization of atherosclerotic lesions at regions of complex geometry such as the branch points, while sparing the straight part of the arterial tree [Giddens et al. 1993].

7 Summary and Conclusions

Shear stress can modulate the structure and function of vascular ECs. Under normal conditions, the modulating influences allow the vessel wall to adapt to changes in pressure and flow to optimize its functional performance. In disease states, however, abnormal responses may disturb the homeostasis and initiate or aggravate pathological processes.

Changes in flow conditions can activate EC mechano-sensors to initiate signal transduction involving Ras and MAPKs such as JNK and ERK and increased expression of MCP-1. The sustained laminar flow (with a definitive forward direction) in the straight part of the arterial tree down-regulates such activation and hence minimizes monocyte entry into the vessel wall. Such laminar flow is also associated with a reduced lipid accumulation in ECs because of (a) the lower LDL permeability due to the up-regulation of growth-arrest genes, and (b) the lower lipid uptake and synthesis due to the down-regulation of SREBP. The reductions of monocyte entry and lipid accumulation induced by long-term laminar shear stress are protective against atherogenesis.

Atherosclerosis occurs primarily in arterial branch points and curved regions. In these lesion-prone regions, the complex flow pattern do not have a clear direction, and they are

associated with increased accumulation of LDL and monocytes and enhanced EC proliferation and apoptosis, all of which are atherogenic.

In conclusion, the laminar shear flow with a definitive direction, which are seen in the straight part of the arterial tree, are athero-protective; whereas disturbed shear flow without a clear direction seen in vascular branch points are atherogenic. Therefore, the presence of a clear direction in shear flow is important for the maintenance of homeostasis of normal vasculature, and the lack of a definitive direction of shear flow at branch points places these regions at risk for atherogenesis.

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