Paradigms of Cellular Mechanotransduction

Since studies have revealed the biochemical effect of mechanical forces on endothelial cells, several paradigms have postulated to understand this transduction of physical stimulus to a chemical response. Currently, three models of cellular mechanotransduction have been proposed:

(1) The "localized model" offers that mechanotransduction occurs via direct conversion of physical force by cell surface molecules into chemical responses.

(2) The "decentralized model" (also known as the "tensegrity cell model") proposes indirect transmission of force by the cytoskeleton to effect subcellular structures and triggering of biochemical signals (Papaioannou *et al.*, 2006).

(3) Evidence suggests that a unified paradigm incorporating both aspects would best serve to interpret the intricacies of mechanotransduction (Figure 1.2.7) (Davies, 2009). Endothelial mechanotransduction in the unified paradigm follows several sequential steps (Davies, 1995):

i. deformation of cell surface,
ii. intracellular transmission of force,
iii. conversion of mechanical force into chemical signals, and
iv. downstream signal transduction and biochemical response.

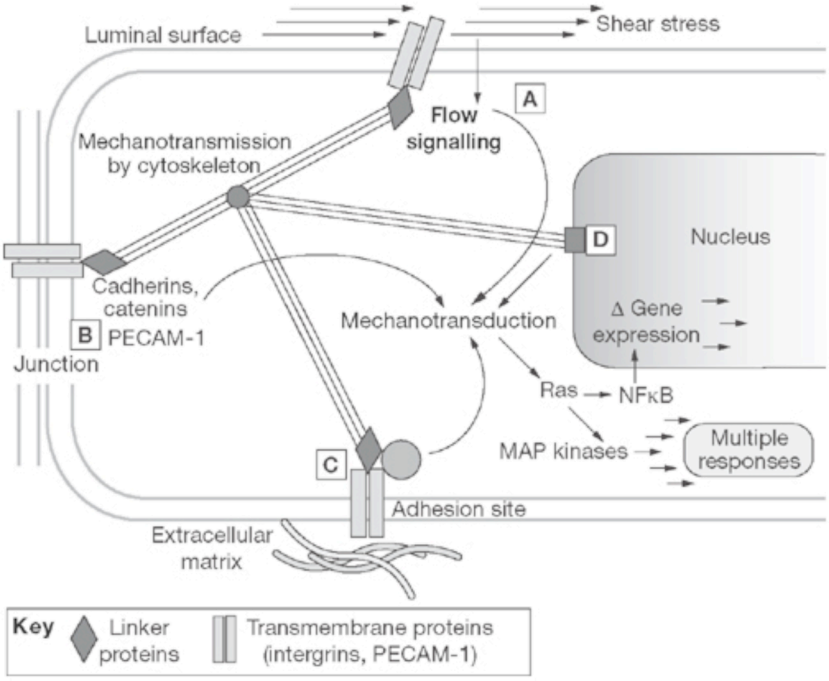

Figure 1.2.7 ***The unified paradigm of mechanotransduction*****.** The unified paradigm incorporates the localized and decentralized models of mechanotransduction whereby cell surface molecules sense and transduce the physical force, and the cytoskeleton transmits the force to subcellular compartments respectively. Adapted from Davies (2008).

While the temporal relationships are not well established, steps (1) and (2) can occur almost simultaneously. Deformation of the endothelial luminal surface affects local membrane structures such as ion channels, G proteins, tyrosine kinase receptors, integrins and cell adhesion molecules. Additionally, distortion of cell shape and bending of the primary cilium transmits the extracellular mechanical force to various subcellular compartments. "True" mechanotransduction occurs at various locations, including changes in molecular conformation, direct effects on ion channels, disassembly of membrane protein complexes and physical changes in integrin dynamics (Davies, 1995). The following sections address the biochemical sensors that play a role in mechanotransduction, and the downstream signaling responses.

Influence of Flow and Shear Stress on the Endothelium

In vivo studies over many years suggest that hemodynamic forces influence arterial wall physiology and pathology in a number of ways (reviewed in (Gimbrone *et al.*, 1997; Davies *et al.*, 1999; Gimbrone *et al.*, 1999; Li *et al.*, 2005)). Laminar flow with physiological level of shear stress (>15 dynes/cm^2) has been extensively studied and shown to have various protective effects on the endothelium. In contrast, oscillatory flow with low shear stress promotes endothelial cell turnover and reduces anti-oxidative responses.

Under laminar flow conditions, endothelial cells have been observed to elongate and orient their major axis with the direction of flow both *in vivo* (Dewey *et al.*, 1981; Levesque & Nerem, 1985; Ives *et al.*, 1986) and *in vitro* (Levesque *et al.*, 1986). This shape reorientation is a dynamic response, involving rearrangement of the F-actin

microfilaments within 24 hours (Sato *et al.*, 1987). Orientation to the direction of flow streamlines the endothelial cell and effectively decreases drag resistance (Barbee *et al.*, 1994; Barbee *et al.*, 1995). Shear stress at a physiological magnitude has also been shown to decrease *in vitro* endothelial cell turnover by decreasing the proliferation rate via inhibition of entry into S-phase (Levesque *et al.*, 1990; DePaola *et al.*, 1992), and reducing apoptosis (Dimmeler *et al.*, 1996; Chiu *et al.*, 1998).

Whereas laminar flow predominates in a straight section of an artery, recirculatory flow patterns with rather extreme variations in shear stress magnitude will exist in a region of branching (Suo *et al.*, 2007). Examples of the spatial variation in shear stress at a bifurcation are the flow at (1) the greater curvature of the aortic arch where it bifurcates into the brachiocephalic, left subclavian and left carotid arteries, and the aorta where it bifurcates into the (2) vertebral, (3) celiac, (4) superior and inferior mesenteric, and (5) renal arteries. At the bifurcation junctions, the stress on the flow divider reaches up to 50 dynes/cm^2, while the mean shear stress on the outer lateral wall will be near zero (Ku *et al.*, 1985). Increased endothelial permeability (Newman *et al.*, 1977) and disruption of endothelial cell alignment (Bjorkerud & Bondjers, 1972; Gutstein *et al.*, 1973) have been observed *in vivo* near these vessel branches and bifurcations. *In vitro* shear stress studies mimicking flow conditions at vessel bifurcations found endothelial cell loss and desquamation, altered morphology with decreased elongation, reduced actin stress fibers, increased proliferation, monocyte attachment and migration across the endothelium, and greater surface expression of the inflammatory molecule VCAM-1 (Walpola *et al.*, 1993; Walpola *et al.*, 1995; Mondy *et al.*, 1997).

In fact, recent technological advances in *in vivo* imaging and computational fluid dynamics have facilitated the study of flow profiles at regions of complex flow while correlating with the expression of inflammatory markers (Suo *et al.*, 2007). The shear stress environment found at these regions of recirculatory flow with low shear stress have long since been purported to be pro-inflammatory, increasing susceptibility of the local vessel wall to endothelial damage, intimal cell proliferation and the development of atherosclerotic lesions (Rittgers *et al.*, 1978).

Mechanosensors of Shear Stress

Mechanosensors of shear stress include integrins, G protein-coupled receptors, ion channels, the glycocalyx, primary cilia, the cytoskeleton, caveolae, adhesion molecules such as PECAM-1, the membrane lipid layer (Osawa *et al.*, 2002; Li *et al.*, 2005) and receptor tyrosine kinases such as vascular endothelial growth factor receptor-2 (VEGFR2) (Chen *et al.*, 1999), Tie1 (Chen-Konak *et al.*, 2003) and Tie2 (Lee & Koh, 2003) (Figure 1.2.8A). Downstream of mechanosensors, the signal is propagated by increases in caveolae formation, and activation of molecules such as protein kinase C, Rho family GTPases, focal adhesion kinases (FAK), MAPK, PI3K, c-Jun-N-terminal kinase (JNK), extracellular related kinase (ERK1/2), tumor necrosis factor-alpha (TNF-α) and nuclear factor-kappaB (nf-κB) (Figure 1.2.8B) (Li *et al.*, 2005).

G Protein-Coupled Receptors

Since the endothelial membrane is directly subjected to shear stress, components of the lipid membrane are likely to perform the function of a shear sensor. G proteins are one

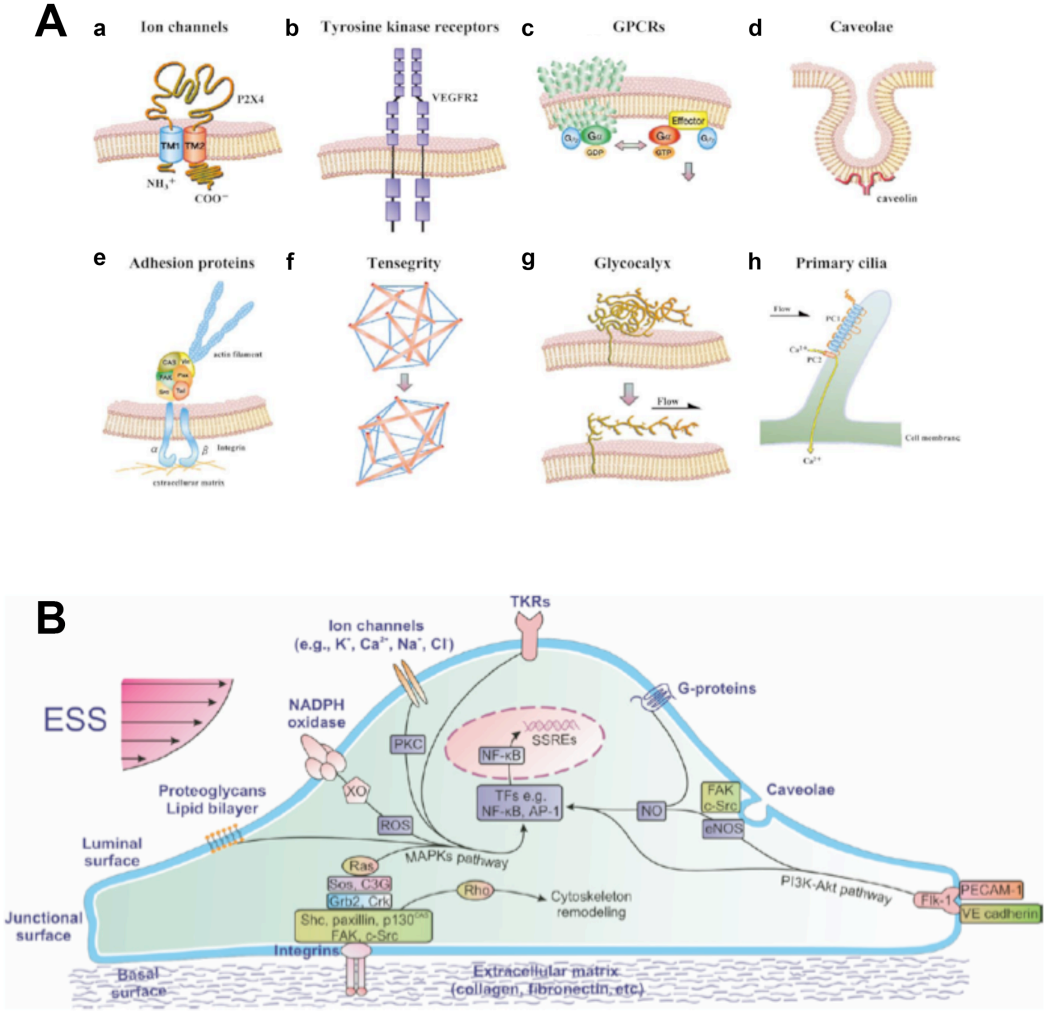

Figure 1.2.8 ***Mechanosensors of shear stress and signaling pathways implicated in mechanotransduction.*** (**A**) Ion channels (a): various types of ion channels including K^+, Cl^- and Ca^{2+} channels; tyrosine kinase receptors (b): VEGFR, Tie1, Tie2; G protein-coupled receptors (c); caveolae (d): membrane microdomain containing various receptors, ion channels and signaling molecules; adhesion proteins (e): including integrins, PECAM; tensegrity (f): an intracellular structure that stabilizes its structure through the use of compression-resistant and tensile elements, such as microtubules and actin filaments; glycocalyx (g): shear stress causes conformational changes under shear stress; primary cilia (h): bending of the primary cilia activates Ca^{2+} channels. (**B**) Sensing of local shear stress by luminal endothelial mechanotransducers. Adapted from Ando (2009) and Chatzizisis (2007).

of the earliest known shear responsive elements (Ohno *et al.*, 1993; Bao *et al.*, 2001), activating within 1 second of flow onset (Gudi *et al.*, 1996). Studies using proteins in artificial phospholipid bilayers in the absence of cytoskeletal elements found that Gαq and Gi respond specifically to alterations in shear stress (Gudi *et al.*, 1998).

PECAM

Besides G proteins, PECAM-1 has also been found to respond shear stress (Harada *et al.*, 1995). PECAM-1 is a glycoprotein expressed in endothelial cells, platelets and leukocytes. It is localized to the cell junctions when endothelial cells are in apposition to one another. Harada et al demonstrated that fluid flow triggers the tyrosine phosphorylation of PECAM-1 within 30 seconds. The direct application of force to PECAM-1 induces its association with the protein tyrosine phosphatase and subsequent ERK activation (Osawa *et al.*, 2002). Similar results were produced when magnetic beads coated with PECAM-1 specific antibodies were used to apply force on the cell surface (Tzima *et al.*, 2005).

Interaction of Mechanosensors – GPCR and PECAM

While PECAM-1 and Gαq co-precipitate as a preformed complex in unstimulated human umbilical vein endothelial cells, onset of flow causes the dissociation of this cell-surface complex (Otte *et al.*, 2009). Tzima et al showed that PECAM-1 together with vascular endothelial cadherin (VECadherin) and VEGFR2 form a mechanosensory complex, and this complex is essential for the alignment of cells to the direction of fluid flow (Tzima *et al.*, 2005). The onset of flow initiates tyrosine phosphorylation of

PECAM-1, and c-Src activation. Subsequently, VECadherin transmits the Src signal to VEGFR2. This ligand independent, Src-dependent, shear stress mediated activation of VEGFR2 binds and phosphorylates the p85 subunit of PI3K. This activity was shown to initiate integrin activation and β-catenin nuclear translocation.

Integrins

Integrins are heterodimers composed of α and β subunits involved in cell-extracellular matrix interactions (ECM). The extracellular domain binds ECM ligands such as fibronectin, vitronectin and collagen, while the intracellular domain interacts with signaling molecules of focal adhesion sites and cytoskeletal proteins (Schwartz & Ginsberg, 2002). Integrins possess unique structural features that enable intracellular signals to regulate affinity of the extracellular domains towards ligands and vice versa (Schwartz *et al.*, 1995). Studies have shown that shear stress induces activation of $\alpha_v\beta_3$ integrins as evidenced by their clustering and binding to the adaptor protein Shc (Tzima *et al.*, 2001; Wang *et al.*, 2002). Activation of integrins occur within 1 minute and association with Shc persists for over 6 hours (Tzima *et al.*, 2001).

Ion channels

Several types of ion channels have been identified that respond to shear stress. Amongst them, the potassium ion (K^+) channel is involved in shear stress induced endothelial NO upregulation (Ohno *et al.*, 1993). Shear stress also activates the calcium ion (Ca^{2+}) channel, increasing the influx of extracellular Ca^{2+} ions in endothelial cells (Helmlinger *et al.*, 1996; Yamamoto *et al.*, 2000; Yamamoto *et al.*, 2000). The

intracellular Ca^{2+} concentration increases within 1 minute of shear stress application, and activates a multitude of Ca^{2+} dependent signaling processes (Ando *et al.*, 1988). *In vivo* genetic deletion studies of the P2X4 Ca^{2+} channel led to loss of NO production and subsequent impaired vasodilation and vascular remodeling (Yamamoto *et al.*, 2006).

Receptor Tyrosine Kinases

The effect of shear stress on several tyrosine kinase receptors have been studied, the most common of which is the vascular endothelial growth factor receptor-2 (VEGFR-2). Onset of flow phosphorylates VEGFR-2 within 1 minute, and causes receptor dimerization and nuclear localization (Chen *et al.*, 1999; Shay-Salit *et al.*, 2002). This ligand independent activation leads to recruitment of PI3K and phosphorylation of Akt, consequently modulating expression of eNOS (Jin *et al.*, 2003). Cyclic strain led to rapid (within 4 minutes) phosphorylation of the PDGFR while disturbed shear stress but not laminar shear stress augmented the expression of the PDGF ligand (Aromatario *et al.*, 1997; Hu *et al.*, 1998; Sumpio *et al.*, 1998; Bao *et al.*, 1999).

Cytoskeleton

Besides individual molecules embedded in the cell membrane, the effect of shear stress can additionally be transduced by the cytoskeleton. This macrostructure is capable of sensing mechanical forces at the surface while transmitting this energy to various cellular compartments including the cell-matrix junction and the nucleus (Bojanowski *et al.*, 1998; Ingber, 1998). Shear stress mediated signaling and transcription was inhibited by disruption of the actin cytoskeleton (Knudsen & Frangos, 1997; Imberti *et al.*, 2000).

Additionally, shear stress was also shown to displace vimentin intermediate filaments (Helmke *et al.*, 2000).

Primary Cilium

The primary cilium is a rod-like, non-motile structure that protrudes from the apical cell membrane expressed in embryonic endothelial cells, human umbilical vein endothelial cells and human aortic endothelial cells (Bystrevskaya *et al.*, 1988; Iomini *et al.*, 2004). Since primary cilia are connected to the cytoskeleton, bending of the cilium transmits the force to the cytoskeletal network. Recent studies have shown that primary cilia sensitize endothelial cells for shear stress and are essential for induction of the Kruppel-like factor 2 (KLF2) transcription factor (Hierck *et al.*, 2008).

Fluid Flow modulates Tie1 Expression and Activity

The most recent additions to the list of shear stress mediators are the Tie family of receptor tyrosine kinases, Tie1 and Tie2. Levels of Tie1 decrease with brief application of physiological laminar flow *in vitro* and its response alters with acute changes in shear stress magnitudes (Chen-Konak *et al.*, 2003). Conversely, disturbed flow conditions *in vitro* upregulates Tie1 promoter activity (Porat *et al.*, 2004). A putative negative shear stress response element has been found to be involved in shear stress mediated suppression of Tie1 expression. However, the role of Tie1 in shear stress induced vascular diseases subject has not been extensively studied.

Tie1 is subject to extracellular proteolytic cleavage by various chemical stimuli (Yabkowitz *et al.*, 1997; Yabkowitz *et al.*, 1999; Marron *et al.*, 2000; Tsiamis *et al.*,

2002) generating a membrane bound receptor fragment comprising the intracellular and transmembrane domains. Production of this cleavage product was increased when bovine aortic endothelial cells were subjected to short intervals of shear stress at physiological levels (Chen-Konak *et al.*, 2003). The truncated Tie1 product persists in the cytosol for several hours (Marron *et al.*, 2000), and was also found to associate with the tyrosine phosphatase and adaptor protein Shp2 (Marron *et al.*, 2000). In consideration of the lack of an identified Tie1 ligand, these reports support a shear stress mediated, ligand independent activation of Tie1.

Since Tie1 has been shown to modulate Tie2 signaling, shear stress regulation of Tie1 function may offer an alternate pathway for regulation of Tie2 activity. Activation of Tie2 by laminar shear stress has been demonstrated to prevent serum-starvation induced apoptosis (Lee & Koh, 2003). Interestingly, both Tie1 holoreceptor and endodomain bind with Tie2 (Marron *et al.*, 2000; Tsiamis *et al.*, 2000), however, neither Tie1 forms are trans-phosphorylated in the presence of activated Tie2. Thus, one of the functions of Tie1 endodomain may be to modulate Tie2 signaling by recruiting proteins to the Tie2 complex.

Biochemical Responses to Shear Stress

Shear Stress Response Elements

Four positive shear stress response elements (SSREs) that promote gene transcription have been reported (Table 1.2.1). The first SSRE was discovered in the promoter region of the PDGF-B gene (Resnick *et al.*, 1993; Khachigian *et al.*, 1995). It is not a consensus-

binding site to any known transcription factors, yet it is capable of binding nf-κB and is necessary for the shear stress-induced upregulation of PDGF-B. Subsequently, studies in flow-induced monocyte chemotactic protein-1 (MCP-1) expression identified a second SSRE in the promoter that is identical to the TPA (tetra-decanoyl phorbol acetate) response element (TRE). This SSRE binds the activator protein-1 (AP-1) transcription factor (which is composed of c-fos and c-Jun dimers) (Shyy *et al.*, 1995). The third SSRE was found in both PDGF-A and tissue factor promoters involving Egr-1 and Sp-1 transcription factors (Khachigian *et al.*, 1997; Lin *et al.*, 1997; Houston *et al.*, 1999). Negative SSREs that repress gene transcription have also been found in VCAM-1 and purine receptor P2X promoters (Korenaga *et al.*, 1997; Korenaga *et al.*, 2001).

Mitogen Activated Protein Kinase Response

Mitogen activated protein kinases (MAPKs) are a group of Ser/Thr kinases activated by phosphorylation at conserved threonine and tyrosine residues (Robinson & Cobb, 1997). Laminar shear stress has been shown to induce PI3K dependent increase in Akt activation (Garcia-Cardena *et al.*, 2000). Laminar flow also induces the phosphorylation of ERK1/2 and p38 but attenuated the activity of JNK within minutes (Li *et al.*, 1996; Surapisitchat *et al.*, 2001). This resulted in an anti-apoptotic effect, which may be modulated in part, by indirect upregulation of survivin (Dimmeler *et al.*, 1998; Kim *et al.*, 2000; Papapetropoulos *et al.*, 2000) and by Akt phosphorylation of BAD and caspase-9 (Datta *et al.*, 1997; Cardone *et al.*, 1998).

Nf-κB and Inflammatory Response

In unstimulated cells, nf-κB is bound in an inactive complex with the inhibitor protein, IκB. Upon stimulation of the nf -κB pathway, IκB is phosphorylated and targeted for degradation by polyubiquitination, thus freeing nf-κB for nuclear translocation and initiation of its transcriptional activities (Hayden & Ghosh, 2004). *In vitro* shear stress systems simulating disturbed flow conditions activated nf-κB (Mohan *et al.*, 1997) and its dependent genes such as ICAM-1 (Walpola *et al.*, 1995), VCAM-1 (Walpola *et al.*, 1995; Nagel *et al.*, 1999), monocyte chemotactic protein-1 (MCP-1) (Shyy *et al.*, 1994), endothelin-1 (ET-1) (Malek *et al.*, 1993), E-selectin and PDGF (Monaco & Paleolog, 2004). The expression of the pro-inflammatory marker, bone morphogenetic protein-4 (BMP-4) is decreased following laminar flow and is also increased by disturbed flow (Sorescu *et al.*, 2003). Expression of BMP-4 causes an increase in ICAM-1 expression and endothelial cell-monocyte adhesion in a nf-κB dependent manner (Sorescu *et al.*, 2003). These molecular biomarkers augment the inflammatory pathway, leading to increased leukocyte adhesion, foam cell formation and atherosclerotic plaque development (Papaioannou *et al.*, 2006).

Endothelial Nitric Oxide Synthase Response

First reported in 1980 by Furchgott and Zawadski (Furchgott & Zawadzki, 1980), biologically produced NO was initially called endothelial-dependent relaxation factor (EDRF). Early studies showed that this vascular relaxation compound was released upon onset of fluid flow *in vitro* (Rubanyi *et al.*, 1986). When EDRF was also found inactivated by superoxide anions (Gryglewski *et al.*, 1986) and protected by superoxide

dismutase (Rubanyi & Vanhoutte, 1986), further pharmacological tests revealed it to be NO (Furchgott & Vanhoutte, 1989).

Endothelial nitric oxide synthase (eNOS) is essential for the synthesis and release of NO, a potent vasodilator, antioxidant and anti-inflammatory molecule, and impaired expression of eNOS is a dominant factor in endothelial dysfunction (Corson *et al.*, 1996; Moncada, 2006). eNOS catalyzes the NADPH-dependent conversion of L-arginine with O_2 to L-citrulline and NO (Rubanyi *et al.*, 1986). NO performs the important function of increasing the blood vessel diameter to relieve shear stress and cyclic strain (Awolesi *et al.*, 1994). Besides vascular relaxation, NO also protects the vessel through inhibition of platelet activation and aggregation, apoptosis and endothelial-dependent monocyte adhesion (Tsao *et al.*, 1996; Dimmeler *et al.*, 1999; Freedman *et al.*, 1999). NO does not suppress endothelial activation chronically; instead it acts as a counter-balancing force only when proatherogenic factors arise (Kuhlencordt *et al.*, 2004). Recent studies have shown that genetic variants in the eNOS gene affecting nitric bioavailability, is an important risk factor in atherosclerosis (Casas *et al.*, 2004).

Human and animal studies have shown that augmentation of cardiac output by exercise and hence increased shear stress can reverse endothelial dysfunction potentially via increased NO release (Green *et al.*, 2004). NO inhibits nf-κB activation (Peng *et al.*, 1995), and it is thought that under disturbed flow conditions, activation of nf-κB decreases eNOS expression (Hajra *et al.*, 2000). Interestingly, under laminar flow conditions, nf-κB binds to the SSRE of eNOS and transiently augments eNOS expression followed by a prolonged stabilization of eNOS mRNA (Davis *et al.*, 2001; Davis *et al.*,

2004). This result suggests a negative feedback loop that acutely increases eNOS and the increased NO level will subsequently decrease nf-κB activity.

Another influence of flow is the synthesis and release of bioactive molecules. One of the most important molecules investigated for its response to flow is NO, a potent vasodilator. Studies have shown that with acute onset of flow, NO expression increases even up to several orders of magnitude (Figure 1.2.9) (Kuchan & Frangos, 1994; Corson *et al.*, 1996). NO expression levels also correlated with increased shear stress magnitudes, remaining high even up to 12 hours after flow onset. Furthermore, this augmentation of NO levels was found to be due to an upregulation of eNOS (Uematsu *et al.*, 1995). These studies show that shear stress is a sensitive regulator of NO and additional experiments have also shown that this regulation of NO release is in part calcium dependent (Kuchan & Frangos, 1994).

Microarray Analyses of Responses to Shear Stress

Laminar flow with high shear stress has been established to have a vasoprotective effect, lowering macromolecule permeability and low-density lipoprotein uptake. It also inhibits leukocyte adhesion, reduces oxidative stress while augmenting expression of antioxidant genes. On the other hand, disturbed flow with low shear stress increases endothelial cell turnover, and raises expression of adhesion molecules, inflammatory and chemokine genes.

Several studies have investigated the effect of shear stress on endothelial cells at the transcription level utilizing broad-spectrum microarrays (Comander *et al.*, 2001; Garcia-Cardena *et al.*, 2001; Garcia-Cardena *et al.*, 2001; Ohura *et al.*, 2003). Ohura et al

A

Table 1. *Relationship between the level of shear stress and the rate of NO_x release*

Shear Stress dyn/cm²	Rate of NO_x Release, nmol · mg⁻¹ · h⁻¹		
	Phase A	*Phase B*	*Phase C*
0	3.3	0.6	0.7
1.8	9.0	14.0	1.7
6	153.0	10.0	2.2
12	106.0	28.0	2.7
25	113.0	28.0	5.2

B

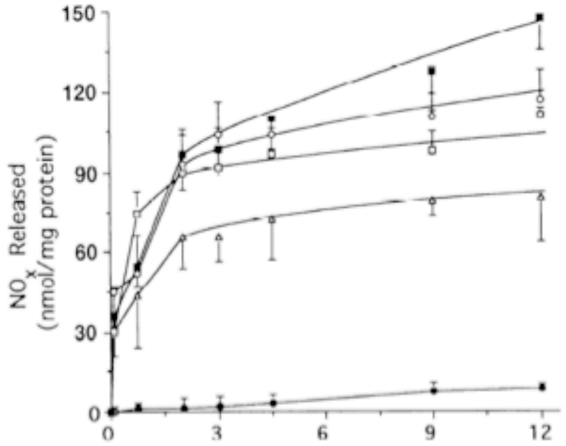

Figure 1.2.9 ***Effect of shear stress on nitric oxide release.*** (**A**) Nitric oxide release over 3 phases A:0-30mins, B: 30min-2hr, C: 2hr-12hr. Cultures were exposed to either 1.8 dynes/cm^2 (△), 6 dynes/cm^2 (□), 12 dynes/cm^2 (○), 25 dynes/cm^2 (■), or control static flow (●). (**B**) Graph of nitric oxide production over time. Note that graph demonstrates biphasic release, an early response rapid increase in nitric oxide is followed by a slower but prolonged release. Adapted from Kuchan (1984).

compared the transcriptional response of human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) when exposed to laminar and turbulent flow conditions. Consistent with results from studies that use the candidate gene approach, Ohura and co-workers found that laminar shear stress upregulates genes involved in antioxidation, anticoagulation and vasodilation (eNOS) and downregulates genes that play a role in DNA synthesis and cell cycle progression. On the other hand, turbulent flow affected the expression of genes that are involved in vascular remodeling, transforming growth factor-β, endothelin-1 and ephrin A1 (Ohura *et al.*, 2003).

Summary

The evolution of shear stress-induced responses in the endothelium is essential to the homeostasis of the vascular trees. Shear stress regulates endothelial cell functions through multiple sensing mechanisms, leading to the activation of a variety of signaling networks, which in turn regulate gene expression. Advances in engineering have facilitated the application of biomathematics to *in vitro* investigations that simulate the effect of fluid flow on endothelial cells, and global gene analyses together with targeted candidate approaches support the idea that laminar flow augments vasoprotective responses while disturbed flow promotes endothelial dysfunction. The pathological effect of disturbed flow on the endothelium *in vivo* is a key element in atherogenesis.

Atherosclerosis occurs primarily in arterial branch points and curved regions. In these lesion-prone regions, disturbed flow promotes accumulation of low-density lipoprotein (LDL) and monocytes, enhances endothelial activation, inflammation and turnover, all of

which are atherogenic. In the next section, we will address atherosclerosis with a perspective from endothelial shear stress.

The Role of Shear Stress in Atherosclerosis

Atherogenesis

Atherosclerosis, the underlying cause of heart attacks and stroke is the leading cause of death in the United States (Heron *et al.*, 2009). The risk factors for this multifactorial disease include genetic predisposition, age, gender, smoking, hyperlipidemia, hypertension, stress, diabetes mellitus, dietary habits and physical inactivity (Ross, 1999).

Atherogenesis is considered to be a form of chronic inflammation triggered by a myriad of risk factors leading to increased endothelial inflammation, turnover and macromolecule permeability (Figure 1.3.1). The increased mitotic and apoptotic activity of endothelial cells (Tricot *et al.*, 2000) as well as morphological changes (Malek *et al.*, 1999) promote the widening of the junctions between endothelial cells, thereby accentuating the sub-endothelial deposition of low density lipoproteins (LDL). Within the intima, LDL is associated with proteoglycans and undergoes oxidative modification by the increased reactive oxidative species (Nakashima *et al.*, 2007). The higher expression of inflammatory chemoattractants leads to an increased migration of leukocytes from the circulation into the arterial wall intima. Once monocytes extravasate into the subendothelium, they undergo functional and structural alterations and differentiate into macrophages, and begin to phagocytize oxidized lipoproteins and cell debris, resulting in the development of lipid-laden, resident foam cells (Ross, 1999). Foam cells produce

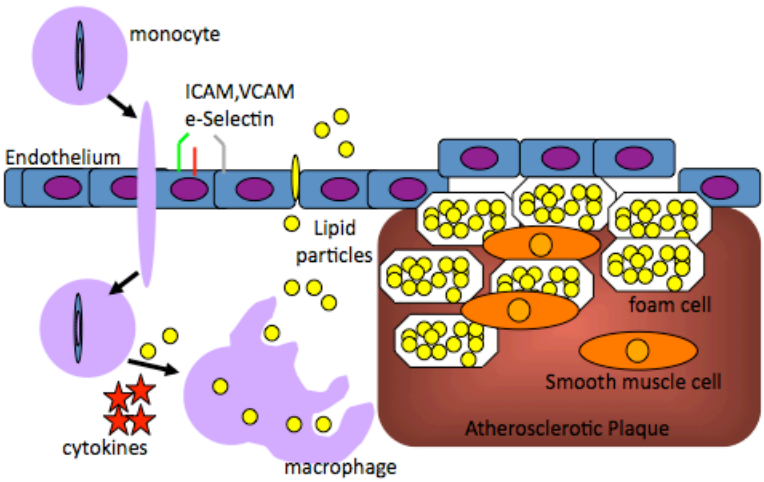

Figure 1.3.1 ***Early events in atherogenesis.*** Endothelial dysfunction facilitates accumulation of lipid particles in the intima. Free radicals oxidation of the lipid particles leads to activation of the endothelium, expressing inflammatory markers and chemoattractants. Expression of luminal cell adhesion molecules attract immune cells and increase their extravasation across the endothelium. Stimulation by cytokines transform the monocytes to macrophages which then phagocytize the oxidized lipid particles. These lipid filled macrophages become resident foam cells which together with migrating smooth muscle cells form the early atherosclerotic plaque.

cytokines, growth factors (eg. PDGF), reactive oxygen species and matrix degrading enzymes (eg. matrix metalloproteases [MMP] and cathepsins). These factors sustain the local inflammation, oxidative stress, matrix remodeling and ultimately lesion expansion. The early vascular lesion, called a fatty streak, worsens with the migration and proliferation of smooth muscle cells from the media. The most advanced and unstable lesion consists of a lipid-rich necrotic core surrounded by an extracellular matrix and is covered by a thin fibrous cap (Ross, 1999). Major clinical complications such as myocardial infarction and stroke arise due to plaque rupture and thrombosis.

Development of atherosclerosis begins in childhood with the development of fatty streaks. This initial lesion presents as a focal thickening of the intima with an increase in smooth muscle cells and extracellular matrix (reviewed in (Davies *et al.*, 1988)). Increased migration of smooth muscle cells causes these early lesions to expand. However, associated with the expansion is increased apoptosis of these smooth muscle cells deep within the fatty streak, leading to further macrophage infiltration and calcification, of the lesions (Kockx *et al.*, 1998). These early lesions will evolve and accumulate connective tissue, evolving into fibrous plaques. Advanced lesions often contain a necrotic lipid-rich core and are self-sustaining via neovascularization both from the luminal and medial surfaces (Stary *et al.*, 1995; Moreno *et al.*, 2004). These advanced plaques are often at risk for focal rupture of the luminal surface, leading to formation of thrombi or release of microemboli into the bloodstream, and further enlarging the plaque by inward hemorrhage. Superimposed thrombus formation on the surface of the ruptured plaques greatly increases the risk of local arterial stenosis and worsens the prognosis (Kumar *et al.*, 2005).

Since the development of apolipoprotein E (ApoE) deficient mice by the laboratories of Dr. Breslow (Plump *et al.*, 1992) and Dr. Maeda (Zhang *et al.*, 1992), ApoE knockout mice have become one of the most commonly used models of atherosclerosis. ApoE is required for the removal of lipoproteins by the liver (Mahley & Huang, 1999). Loss of ApoE in mice mimics human type III hyperlipidemia (Ghiselli *et al.*, 1981) by causing severe hypercholesterolemia even on a regular chow diet. Similar to human atherosclerosis, ApoE deficient mice develop spontaneous atherosclerotic lesions at the aortic sinus and pervasive fibrous plaques at multiple aortic and arterial branch points (Nakashima *et al.*, 1994; Reddick *et al.*, 1994). While on normal chow diet, fatty streaks appear after 10 weeks, smooth muscle cell and foam cell laden intermediate lesions appear after 15 weeks and advanced fibrous plaques appear after 20 weeks (reviewed in (Fazio & Linton, 2001)). Feeding these mice a Western diet with higher cholesterol and fat content will result in severe hypercholesterolemia and significantly accelerate the progression of atherosclerosis (Nakashima *et al.*, 1994; Reddick *et al.*, 1994).

Historical Perspective: Investigation of the Role of Shear Stress in Atherosclerosis

The study of fluid flow in arteries began as early as the 18th century with Leonhard Euler and Thomas Young (Caro, 2009). However, interest in the application of findings to vascular pathologies really only started in the mid 19th century. After several iterations of names used in the early years for describing fluid dynamics, “hemodynamics” was first instituted as the title of the field in 1968 (McDonald, 1968).

Dr. Fry’s laboratory was amongst the first to demonstrate the alignment of endothelial cells to the direction of blood flow (Flaherty *et al.*, 1972) and the changes in permeability

of the endothelium with shear stress alterations (Fry, 1969). As summarized by Dr. Texon, early reports almost 4 decades ago correlated atherosclerotic lesions found at autopsy with their localization in the circulatory system as determined by hydraulic forces (Texon, 1957; Texon, 1960).

Dr. McDonald (McDonald, 1955; McDonald, 1968) along with Dr. Duguid and Dr. Robertson (Duguid & Robertson, 1957) were the first to speculate at a correlation between common atherosclerotic regions and the local shear forces. Based on these initial reports, Dr. Mitchell and Dr. Schwartz supposed that the elevated shear stress would cause mechanical damage to the endothelium and thus suggested that areas of high shear stress are likely to show a high prevalence of fatty streaking (Caro, 2009). On the contrary, as investigations in the following years show, this early concept of mechanical damage was inaccurate. Caro et al showed that the inner walls of the flow divider (Figure 1.3.2) developed fewer lesions than the outer wall, implying less involvement of the regions experiencing higher shear stress (Caro *et al.*, 1969; Caro *et al.*, 1971). In light of these initial findings, a multitude of studies went on to show in various anatomical regions of human cadavers, a predilection of atherosclerotic plaques for regions that experience low shear stress (Caro *et al.*, 1969; Kjaernes *et al.*, 1981; Grottum *et al.*, 1983; Svindland, 1983). Using an *in vitro* model of pulsatile flow, Friedman et al measured fluid flow velocities at the wall of casts made from human aortic bifurcations. They reported an inverse relation between shear stress at the wall and the size of lesions (Friedman *et al.*, 1981). Zarins et al correlated fluid flow profiles in comparable scale models with the distribution of plaques in autopsied human carotid artery bifurcations (Zarins *et al.*, 1983). In complement with Dr. Friedman's results, they showed that

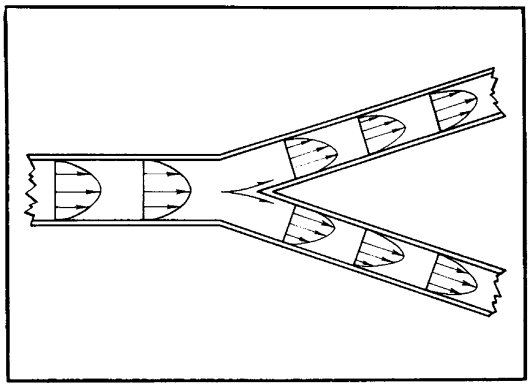

Figure 1.3.2 ***Illustration of shear stress profile at a flow divider.*** The parabolic nature of shear stress profiles creates areas of laminar flow with high shear stress at the walls of the flow divider. Adapted from Texon (1965)

regions of laminar flow with moderate to high shear stress were relatively spared of atherosclerotic plaques.

Furthermore, Caro et al also proposed a "mass transfer mechanism" involving the effect of local shear stress on (1) the supply of raw materials for lipid synthesis, (2) the clearance of lipids synthesized or modified at the arterial wall, and (3) the gradual accumulation of lipids (Caro *et al.*, 1971).

The belief was held for many decades that acute mechanical damage to the arterial wall is the underlying cause of atherogenesis. It is now widely accepted that atherosclerosis develops in regions of low shear stress or disturbed flow through a process of chronic inflammation. In addition to genetic and systemic risk factors, atherosclerosis has biomechanical predilections towards regions of reduced laminar flow or turbulent (Tricot *et al.*, 2000). The endothelium is the frontline exposed to local changes in shear stress and endothelial dysfunction is widely accepted as a crucial early event in the atherogenesis. Although wall damage due to shear stress can occur, it is only in the rare instance that the upper limit of vessel tolerance is exceeded (Fry, 1968).

Increased inflammation, endothelial turnover and macromolecule permeability in regions of recirculating flow has been implicated in atherosclerosis (Wright, 1972; Chiu *et al.*, 1998). Laminar flow with high shear stress (at physiologic levels) promotes endothelial survival, decreases activation and macromolecular permeability. On the other hand, low shear stress or disturbed flow augment endothelial turnover, increase inflammatory marker expression and promote endothelial activation. In light of the infinite permutations in genetic and environmental risk factors, the discovery of the role

of shear stress in atherosclerosis provides key information in our understanding of this debilitating disease.

Shear Stress and the Focal origin of Atherosclerosis

Atherosclerotic lesions are preferentially localized to the outer walls of arterial branches and to the inner curvatures of tortuous vessels, where the local flow is disturbed (Figure 1.3.3) (Caro *et al.*, 1971; Zarins *et al.*, 1983; Ku *et al.*, 1985; Asakura & Karino, 1990). These atherogenic regions exhibit directional changes with flow separation and reattachment, creating a localized environment with a low average shear stress magnitude. Direct measurements and analyses of computational fluid dynamics models of lesion-prone areas have revealed that shear stress in these regions are on the order of ± 4 dynes/cm^2 which is much lower than the values of >10 dynes/cm^2 in the lesion-free areas (Figure 1.3.4) (Malek *et al.*, 1999). Magnetic resonance imaging and ultrasound studies of the aortic arch in humans, mice and pigs have identified a complex series of flow-reversal events in the inner curvature of the arch (Figure 1.3.5) where endothelial cells display a polygonal morphology (Suo *et al.*, 2007). Beyond the arch in the descending thoracic aorta, the flow of blood resumes a laminar profile, as signified by realignment of the endothelial cells (Suo *et al.*, 2007). The sparing of high net flow regions to lesion formation, and the susceptibility of regions with disturbed flow suggest that laminar flow with high shear stress protects against atherosclerosis, whereas disturbed flow with low shear stress act as detrimental mechanical stimuli contributing to atherogenesis (Nerem, 1992; Malek *et al.*, 1999; Wootton & Ku, 1999; Gimbrone *et al.*, 2000).

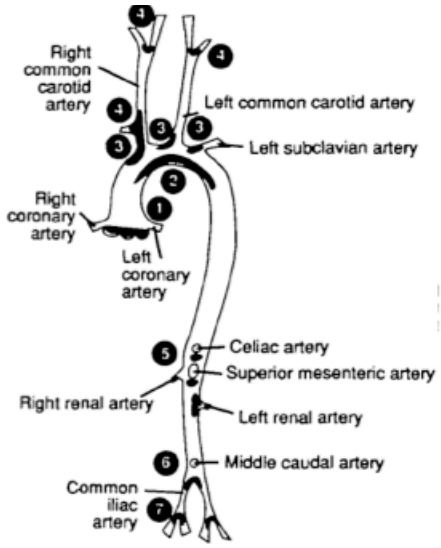

Figure 1.3.3 ***Topograph of common atherosclerotic plaque locations in the aorta.*** Atherosclerotic lesions have a predilection for locations of disturbed flow such as, (1) aortic sinus, (2) inner curvature of aortic arch, (3) aortic arch branches, (4) carotid artery bifurcations, (5) abdominal aorta branches (celiac, superior mesenteric and renal), (6) middle caudal artery, and (7) common iliac arteries.

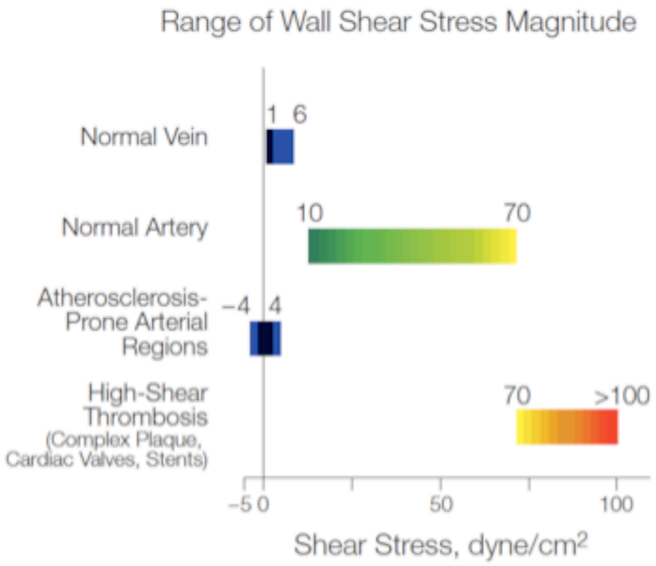

Figure 1.3.4 ***Illustration of typical shear stress magnitudes in human vasculature.*** Normal shear stress values for veins, arteries range from 1 to 70 dynes/cm^2. Low oscillating values are characteristic of atherosclerosis prone regions, whereas complex lesions and stent struts present regions of abnormally high shear stress magnitudes. Modified from Malek (1999).

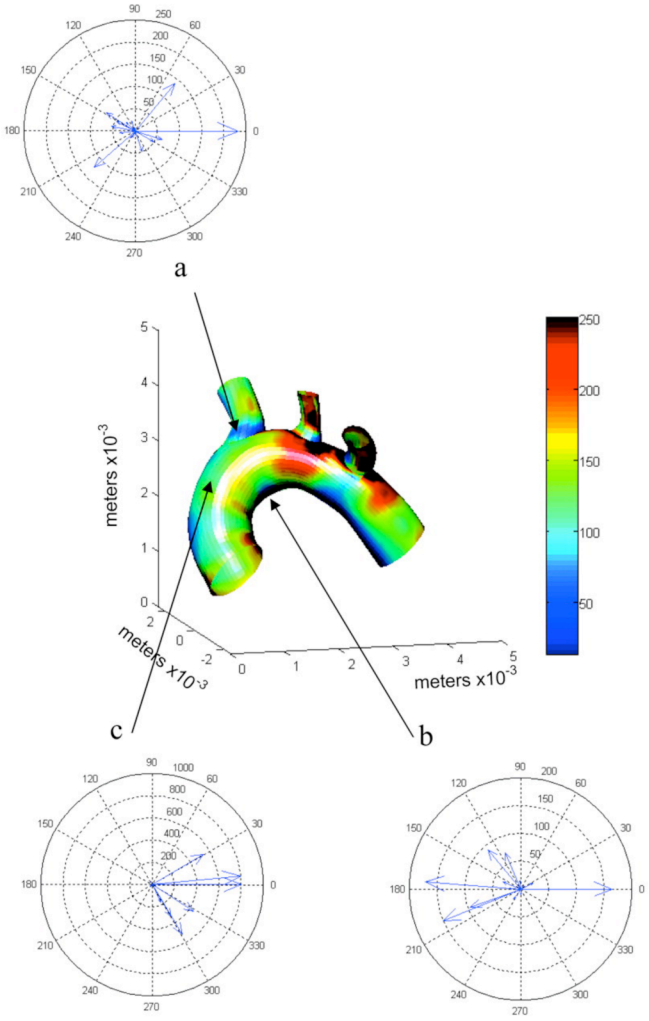

Figure 1.3.5 ***Computational fluid dynamics model of shear stress magnitudes.*** Model of shear stress magnitudes gathered from ultrasound studies. Regions in red experience the highest shear stress whereas areas in blue report the lowest values. Vector maps illustrate magnitude of shear stress with respect to direction. Note that region (b) the inner curvature of the aortic arch displays the widest range of directional changes together with the highest shear stress magnitudes. Adapted from Suo (2007)

Shear Stress Regulates Inflammation

Inflammatory Markers

Oscillatory flow *in vitro* has been shown to induce the expression of inflammatory markers involved in atherogenesis such as ICAM-1 (Sorescu *et al.*, 2003), E-selectin (Chappell *et al.*, 1998) and also the vasoconstrictor ET-1 (Ziegler *et al.*, 1998). In contrast, *in vitro* laminar flow has little effect on E-selectin and ICAM-1 expression (Nagel *et al.*, 1994; Morigi *et al.*, 1995) and causes a downregulation of ET-1 and VCAM-1 (Malek *et al.*, 1993; Ohtsuka *et al.*, 1993; Korenaga *et al.*, 1997). The pro-inflammatory molecule BMP4 also increases in response to oscillatory flow but decreases with laminar shear stress (Sorescu *et al.*, 2003). Iiyama et al showed that VCAM-1 and ICAM-1 mRNA levels were increased in both the LDL receptor knockout and the ApoE knockout mouse models (Iiyama *et al.*, 1999; Suo *et al.*, 2007). Assessing the aortas of wild-type mice and rabbits, they showed that both these inflammatory markers were expressed at the inner curvature of the aortic arch and the outer wall of arterial branch points, all regions that are exposed to atherogenic shear stress (Figure 1.3.6). Notably, staining of both VCAM-1 and ICAM-1 was found to be most intense at the shoulder of lesions (Iiyama *et al.*, 1999) providing evidence that the changes in vessel geometry create a microenvironment of abnormal shear stress that then augment the local inflammation.

Sustained laminar shear stress *in vitro* attenuates the expression of MCP-1 and various pro-inflammatory or pro-proliferative genes to below the static control level

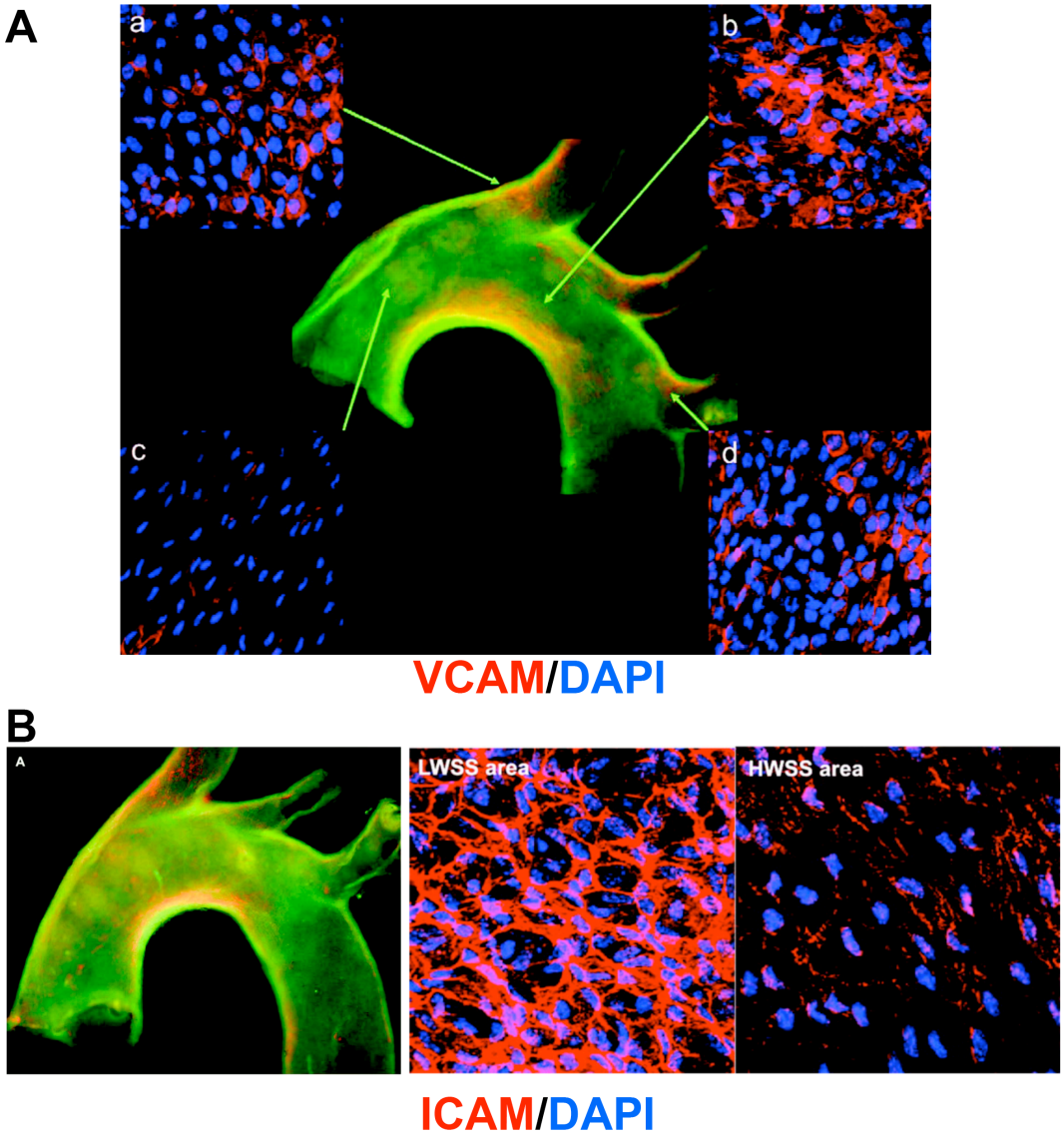

Figure 1.3.6 ***Expression of inflammatory markers at regions of disturbed flow.*** (**A**) VCAM expression is augmented at the proximal wall of aortic arch branch points and also the inner curvature of the aortic arch. (**B**) Increased ICAM expression at regions of low shear stress. Adapted from Suo (2007).

High wall shear stress, hwss; Low wall shear stress, lwss.

(Shyy *et al.*, 1995; Li *et al.*, 1996). Hsiai et al showed that the induction of MCP-1 expression by oxidative stress is attenuated by laminar flow and augmented by disturbed flow (Hsiai *et al.*, 2003). Immunohistochemistry has shown a predilection of MCP-1 expression near intercostal artery orifices (Chien, 2003) and aortic branch points (Malinauskas *et al.*, 1995). The functional consequence of MCP-1 upregulation is an increase in monocyte attraction into the vessel wall and thus promoting atherosclerosis.

Increased Pro-Inflammatory Cell Residence

Low shear stress surrounding the stagnation point of recirculatory flow may also allow prolonged residence times for circulating pro-inflammatory cells, increasing adherance to the endothelial monolayer (Glagov *et al.*, 1988). Zhu et al demonstrated that low shear stress magnitude augmented adhesion of monocytes to endothelial cells (Zhu *et al.*, 2004). Hence, besides potentiating the expression of inflammatory molecules, atherogenic shear also facilitates the physical environment for attachment of leukocytes to the endothelium.

Reactive Oxygen Species

Disturbed flow also mediates the production of reactive oxygen species by increasing gene expression and post-transcriptional activities of the major oxidative enzymes (eg. NADPH and xanthine oxidase at endothelial cell membranes (McNally *et al.*, 2003). The local oxidative stress is further augmented as disturbed flow downregulates the intracellular reactive oxygen species such as manganese superoxide dismutase (MnSOD) and glutathione (Brooks *et al.*, 2002).

Kruppel-like factor-2

The endothelial transcription factor Kruppel-like factor-2 (KLF-2) is only induced by flow (Dekker *et al.*, 2002). Laminar but not oscillatory flow upregulates expression of KLF-2, a shear stress specific transcription factor that increases anti-inflammatory activity (Dekker *et al.*, 2002; Wang *et al.*, 2006). Several studies also demonstrated that KLF-2 is exclusively expressed only in regions that do not develop atherosclerosis, suggesting that KLF-2 may play a critical role underpinning the molecular basis of laminar flow induced protection against atherosclerosis (Garcia-Cardena *et al.*, 2001; Dekker *et al.*, 2002; Parmar *et al.*, 2006). Parmar et al further postulated that KLF-2 might regulate up to one-third of all shear stress activated genes (Parmar *et al.*, 2006).

Regulation of Endothelial Cell Turnover by Shear Stress

DNA microarray studies on the effects of laminar flow on human aortic endothelial cells showed that genes related to endothelial cell proliferation and inflammation were downregulated whereas genes related to survival and angiogenesis were upregulated (Zhao *et al.*, 2002).

Proliferation

Earlier studies have shown that laminar flow causes a reduction of endothelial cell proliferation rate in a dose-related manner (Levesque *et al.*, 1990). Laminar shear stress increases the expression of growth arrest proteins (Lin *et al.*, 2000) while lowering the rate of DNA synthesis (Mitsumata *et al.*, 1993) thus leading to cell cycle arrest. Recent

experiments show that laminar flow reduced Cdk kinase activity thus reducing the number of cells entering into G0 or G1 phase (Akimoto *et al.*, 2000; Lin *et al.*, 2000).

The retinoblastoma protein (Rb) is also regulated by shear stress. Rb is a nuclear phosphoprotein that binds to DNA to regulate cell cycle progression through its phosphorylation status (Bartek *et al.*, 1996). Laminar shear stress decreases Rb phosphorylation, allowing it to bind transcription factors essential for DNA synthesis and hence inhibit cell proliferation (Lin *et al.*, 2000). In concert with several other studies (Li *et al.*, 2005), these results suggest that laminar shear stress keeps the endothelial cell gene expression profile in a non-proliferative and non-inflammatory state.

In contrast, *in vitro* studies using a parallel plate step flow system showed a significantly higher rate of DNA synthesis in endothelial cells exposed to disturbed flow than those experiencing laminar flow (Chiu *et al.*, 1998). Disturbed flow also increased BrdU incorporation in endothelial cells when compared to those subjected to laminar flow (Chien, 2003). The same distribution pattern is seen for the activation of signaling molecules for proliferation such as ERK and the increase in BrdU incorporation can be inhibited blocked by the ERK inhibitor PD98059. In addition, Conklin et al (Conklin *et al.*, 2002) demonstrated that low shear stress *in vitro* increases expression of VEGF, an endothelial-specific mitogen that also activates the ERK pathway. These results indicate that the disturbed flow pattern at arterial branch points stimulates cell proliferation via ERK activation in contrast to the upregulation of growth arrest genes when endothelial cells are exposed to laminar flow.

Apoptosis

Dimmeler et al have shown that laminar flow suppresses endothelial apoptosis induced by TNF-α, oxygen radicals, oxidized LDL, and serum depletion (Dimmeler *et al.*, 1997; Dimmeler *et al.*, 1997). They also showed that this shear stress protection from apoptosis was blocked by either the inhibition of NO production with L-NG-monomethyl arginine (LNMA), or the inhibition of Akt phosphorylation by PI3K inhibitors, or the expression of dominant-negative Akt. They subsequently showed that shear stress activates the Akt pathway, in turn causing the serine phosphorylation of eNOS and the production of NO in endothelial cells (Dimmeler *et al.*, 1998). Hence, the anti-apoptotic effect of laminar flow is mediated in part, by eNOS activity.

Fluid flow modulates Macromolecular Permeability at Endothelial Junctions

Endothelial intercellular junctions do not normally allow transmigration of macromolecules such as LDL. The integrity of the monolayer is temporarily compromised when a cell undergoes replication. Studies have provided evidence that endothelial cell turnover, including mitosis and apoptosis are associated with increased permeability of the endothelium to macromolecules such as LDL and albumin (Caplan & Schwartz, 1973; Bell *et al.*, 1974; Stemerman *et al.*, 1986; Lin *et al.*, 1988; Chen *et al.*, 1995).

In the thoracic aorta, mitotic cells and leaky areas permeable to Evans Blue albumin (EBA) were notably distributed around the orifices of intercostal arteries (Chien, 2003). In a normal rabbit aorta, these lesion-prone branch points were four times more permeable to macromolecules than that of the lesion-resistant non-branching areas

(Schwenke & Carew, 1989). These distributions of mitosis and EBA-permissive areas were similar to the patterns of lipid accumulation in cholesterol-fed rabbits (Schwenke & Carew, 1989) and of human atherosclerosis lesions (Texon, 1995).

Shear stress also modulates intercellular junction proteins such as connexins (Kwak *et al.*, 2002), PECAM-1 (Tzima *et al.*, 2005) and VECadherin (Miao *et al.*, 2005). Immunostaining of VECadherin in endothelial cells exposed to laminar flow *in vitro* showed robust and continuous cell border expression similar to those observed in the descending thoracic and abdominal aorta (Miao *et al.*, 2005). In contrast, cells exposed to disturbed flow *in vitro* displayed weak and discontinuous border staining mirroring those seen in the aortic arch (Miao *et al.*, 2005). Additionally, experiments that partially constricted the rat abdominal aorta showed robust VECadherin staining at areas of laminar flow, but no expression was found in the endothelial cell borders of the post-stenotic sites where the flow is disturbed.

Dai et al further showed that the expression of connexin 37 and 40 are upregulated in regions of the aorta experiencing laminar flow but were not detectable at branch points exposed to disturbed flow (Dai *et al.*, 2004). Also, endothelial cells in regions of disturbed flow show a loss of peripheral F-actin microfilaments at the borders (Chiu *et al.*, 1998). These effects of disturbed flow on the redistribution of gap junctions and cytoskeletal proteins may contribute to intercellular widening, and consequently an increase in macromolecular permeability.

Shear Stress regulates Lipid Metabolism

The activity of sterol regulatory element binding protein 1 (SREBP1) can increase the expression of LDL receptor, cholesterol synthase and fatty acid synthase, thus increasing the intracellular sterol level. Disturbed flow causes a sustained activation of SREBP1 and expression of genes that impair endothelial cell lipid homeostasis (Liu *et al.*, 2002). Using a parallel plate flow model, Liu et al showed that disturbed flow increased the proteolytic cleavage of SREBP1 and translocation of the transcription factor endodomain into the nucleus. As a result, disturbed flow augmented transcription of the low-density lipoprotein receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, and fatty acid synthase, all of which promote lipid accumulation. Using a step flow model, they showed that laminar flow only causes a transient activation of SREBP1, but disturbed flow causes a sustained activation. Hence, atherogenic shear stress increases the bioavailability of LDL in the local environment.

The Self-Perpetuating Cycle: Atherogenic Shear Stress and Lesion growth

A developing atherosclerotic lesion may itself alter the local flow profile on the endothelium (Figure 1.3.7). With expansion of the lesion a substantial stenosis occurs, creating an area of lowered shear stress upstream and disturbed shear stress immediately downstream. Cheng et al previously showed that lowered shear stress incites the development of unstable plaques whereas disturbed flow promotes the formation of stable lesions (Cheng *et al.*, 2006). Additionally, stents placed to maintain vessel patency also create microenvironments of disturbed flow around the stent struts (Moore & Berry, 2002; Duraiswamy *et al.*, 2005). *In vitro* and *in vivo* studies of endothelial cells have

demonstrated that these environments promote pro-inflammatory gene expression that increases atherosclerosis susceptibility (Davies *et al.*, 1999; Garcia-Cardena *et al.*, 2001),

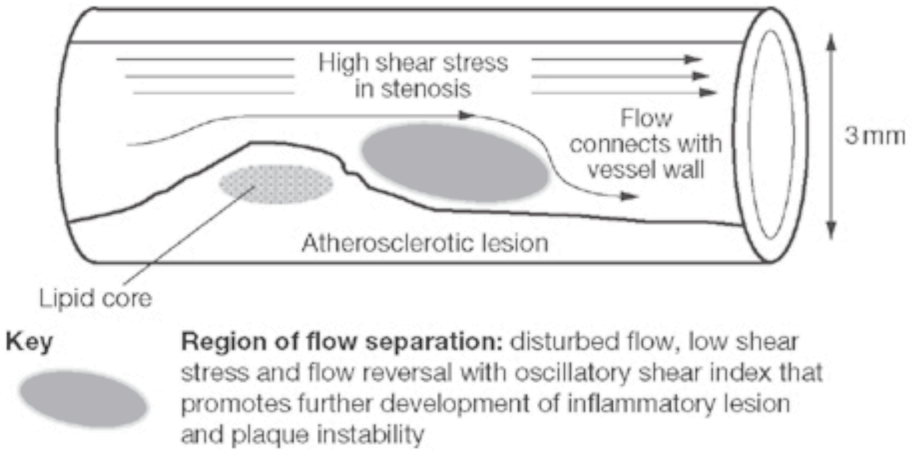

Figure 1.3.7 ***Alteration of the local shear stress profile by a developing lesion.*** Adapted from Davies (2008).

plaque growth and instability (Libby, 2000) and increased rick of thrombosis (Folie & McIntire, 1989).

The Fate of Early Lesion Progression

For the purposes of classification, the American Heart Association groups atherosclerotic lesions as follows (Kumar *et al.*, 2005):

Type I: (initial lesion) isolated macrophages and foam cells

Type II: (fatty streaks) mainly intracellular lipid accumulation

Type III: (intermediate lesion) Type II changes and small extracellular lipid pools

Type IV: (atheroma lesion) Type II changes and core of extracellular lipid

Type V: (fibroatheroma lesion) lipid core and fibrotic layer; or multiple lipid cores and fibrotic layers; or mainly calcific or mainly fibrotic

Type VI: (complicated lesion) surface defects, hemorrhages and/or hematomas, thrombus formation

A number of intermediate lesions acquire a fibrous cap and evolve to early fibroatheromas. Studies suggest that this transition is due in part to disturbed flow and the subsequent local inflammation, whereby the internal elastic lamina undergoes degradation by foam cell-derived proteases (MMP-2, -9, and cathepsins) (Chatzizisis *et al.*, 2008). These compromises in the internal elastic lamina provide an entry point for the smooth muscle cells that reside in the media (Bentzon *et al.*, 2006). Disturbed flow further promotes smooth muscle cell migration by enhancing the expression of growth

promoters (PDGF, endothelin-1, and VEGF), and attenuating the expression of growth inhibitors (NO, TGF-β, and plasminogen activator inhibitor-1 [PAI-1]) (Cheng *et al.*, 2006; Chatzizisis *et al.*, 2007). Once in the intima, smooth muscle cells proliferate under the effect of growth factors secreted by foam cells and endothelial cells, and they also differentiate to a more synthetic phenotype, increasing extracellular matrix component production and hence expanding the lesion. The smooth muscle cells surround the core of the lipid-rich foam cells, produce extracellular matrix and create a fibrous cap, thus creating the Type IV early fibroatheroma (Stary *et al.*, 1995; Virmani *et al.*, 2000).

The Role of Disturbed Shear Stress in Lesion Progression

Following their formation, a portion of early fibroatheromas will develop into high-risk plaques, whereas others will evolve into fibrous stenotic plaques or remain quiescent (Chatzizisis *et al.*, 2007). High-risk plaques are typically thin-cap fibroatheromas (TCFA) composed of a thin, fibrous cap covering a densely neovascularized, necrotic lipid core (Virmani *et al.*, 2000). These plaques usually do not narrow the lumen but promote expansive remodeling of the vessel, however, they are associated with a high risk of sudden rupture that causes 60-70% of coronary syndromes (Burke *et al.*, 2002; Varnava *et al.*, 2002; Chatzizisis *et al.*, 2007)}. Studies have shown that disturbed flow enhances inflammation, especially at the base of the plaque (Chatzizisis *et al.*, 2008). In this setting the augmented release of matrix proteases severely degrades the internal elastic lamina, and inflammation spreads to the media. The extracellular matrix of the media also undergoes degradation thereby promoting wall expansion and accommodating the expanding plaque (Sipahi *et al.*, 2006). This expansive remodeling is perpetuated as local

flow dynamics are altered thereby fostering further lipid accumulation and inflammation and the rapid transformation of an early fibroatheroma into a TCFA.

Quiescent plaques are minimally stenotic plaques characterized by limited inflammation and are asymptomatic (MacIsaac *et al.*, 1993). Quiescent lesions also develop in the presence of disturbed flow but do not acquire the same degree of lipid accumulation and inflammation as TCFAs (Chatzizisis *et al.*, 2007). With reduced inflammation, the degree of internal elastic lamina degradation is reduced, and there is less expansive remodeling, hence these lesions protrude more into the lumen. As local hemodynamic factors change or atherosclerotic stimuli are enhanced, the process of inflammation may transform the quiescent lesion into a TCFA (Wentzel *et al.*, 2003).

Stenotic plaques are stable fibroproliferative plaques with limited inflammation and are characterized by a thick, collagen-rich fibrous cap, covering a relatively small lipid core (Virmani *et al.*, 2000; Varnava *et al.*, 2002). Many stable fibroproliferative plaques represent the final stage of TCFAs that have repeatedly ruptured and healed (Burke *et al.*, 2002). Alternatively, stenotic lesions may also develop from early fibroatheromas that undergo less inflammation (Feldman *et al.*, 2006). These lesions occlude the vessel and are responsible for chronic stable angina. The severity of disturbed flow may play a role in the degree of inflammation regulating the differentiation of the early fibroatheroma either into a quiescent lesion or a TCFA (Chatzizisis *et al.*, 2007).

Potential Role of Tie1 in Atherosclerosis

Tie1 expression correlates distinctively to vascular regions exposed to disturbed flow in both physiological and pathological conditions (Figure 1.3.8) (Porat *et al.*, 2004). Tie1

promoter activity is increased downstream of branches and at bifurcations where endothelial cells experience wide variations in hemodynamic force. These regions of high

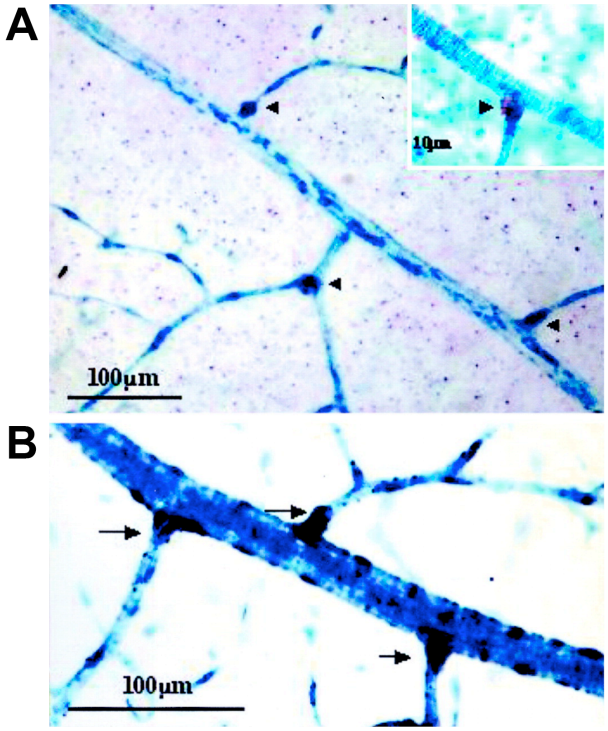

Figure 1.3.8 ***Tie1 promoter activity in mouse retinal arteries.*** (**A**) Tie1-LacZ expression is increased at branch points of the mouse retina. (**B**) Higher magnification.

shear stress gradients correspond exquisitely with areas of disturbed flow such as the inner leaflet of the aortic valve, the junction of vein-to-artery grafts and at the shoulder of atherosclerotic plaques. The effect of fluid shear on Tie1 has also been demonstrated *in vitro* whereby disturbed flow upregulates Tie1 expression (Chen-Konak *et al.*, 2003; Porat *et al.*, 2004). However, the role of Tie1 at these locales of atherogenic shear stress has not been investigated.

Summary: Shear Stress - Tie1 - Atherosclerosis

Atherosclerosis, while associated with many systemic risk factors (including genetics, hyperlipidemia, hypertension, smoking, diabetes and obesity), has a predilection for development at the outer walls of blood vessel bifurcations and at curvatures of the blood vessel. In these regions exposed to atherogenic flow, shear stress is significantly lower in magnitude and exhibits dramatic changes in flow direction. In contrast, straight regions of the arterial tree experiencing laminar flow do not develop atherosclerosis. This striking correlation between regional hemodynamics and atherosclerosis has sparked many investigations to elucidate a mechanistic role for hemodynamic factors, specifically the different flow profiles and alterations in shear stress magnitudes, in the pathogenesis of atherosclerosis.

Shear stress can modulate the structure and function of vascular endothelial cells. Alterations in blood flow can activate endothelial cell mechanosensors to initiate signal transduction involving Ras and MAPKs such as JNK and ERK and regulate expression of pro-inflammatory molecules such as MCP-1, ICAM-1 and VCAM-1. Laminar flow in the straight part of the arterial tree downregulates such activation and hence reduces

monocyte entry into the subendothelium. Several genes that are anti-inflammatory, anti-apoptotic, anti-oxidant and ant-coagulant are upregulated by laminar flow. Such laminar flow is also associated with lowered lipid accumulation due to a reduced lipid permeability, uptake and synthesis. These reductions in monocyte invasion and lipid accumulation induced by laminar flow are protective against atherosclerosis (Table 1.3.1).

Broad-spectrum microarray profiling of endothelial cells exposed to disturbed flow have higher levels of pro-inflammatory, pro-coagulant, proliferation and pro-apoptotic genes, all of which are atherogenic. Hence, laminar flow is atheroprotective, whereas disturbed flow seen at branch points and curvatures is atherogenic.

In summary, the sequence of events that contribute to the focal nature of atherosclerosis involves local hemodynamic factors, endothelial cell turnover (apoptosis and mitosis), local enhancement of LDL permeability, monocyte entry and focal lipid accumulation (Figure 1.3.9). The role of Tie1 in this complex pathogenic process has not been clearly defined.

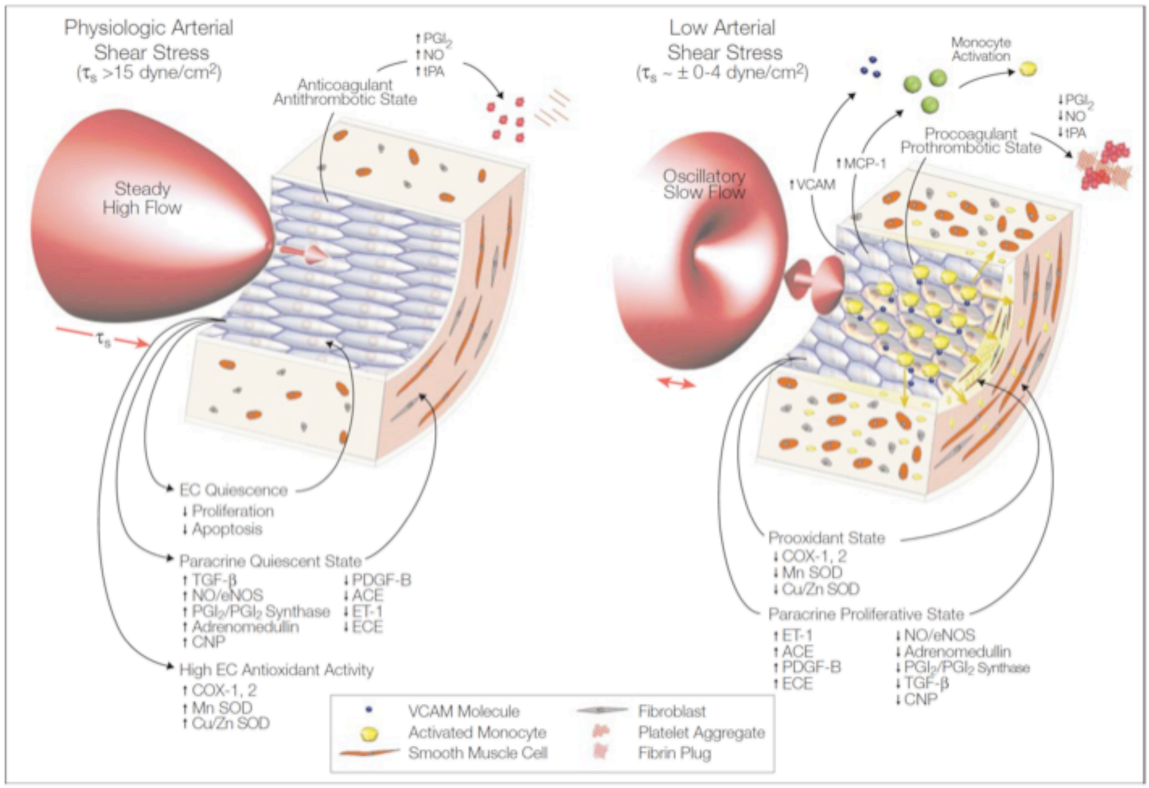

Figure 1.3.9 ***Model of the role of shear stress in atherogenesis.*** Illustration of the change in the endothelial role from atheroprotective (left) to atherogenic (right) induced by the local hemodynamic conditions. Laminar flow with high shear stress promotes the expression and release of vasoprotective molecules. Oscillatory flow with low shear stress conditions found in atherosclerosis-prone regions result in recruitment and activation of monocytes, increased platelet activation, increased vasoconstriction and paracrine growth stimulation of vessel wall components, increased oxidant state, and increased endothelial cell turnover. Adapted from Malek (1999).

Shear stress,τ_s; nitric oxide, NO; endothelial cell, EC; endothelial nitric oxide synthase, eNOS.

Aims of Dissertation

Extensive research has shown that shear stress is a key factor in atherogenesis and also an important modulator of vascular endothelial biology, whereas recent studies have indicated a role for Tie1 endothelial cell activation. We therefore hypothesized that Tie1 plays an essential role in endothelial response to atherogenic shear. To test this hypothesis we propose to:

Aim 1. *Define the expression pattern of Tie1 in the macrovasculature and in modified flow regions.* Tie1 promoter-LacZ reporter mice will be dissected and studied to determine locations of the aorta where Tie1 is expressed and correlated with known local shear stress profiles. Shear stress modifying casts will be implanted in mouse carotid arteries and the Tie1 promoter activity will be assessed with respect to the specific flow profile.

Aim 2. *Define the effect of Tie1 attenuation in atherosclerosis progression. Tie1 heterozygous mice will be crossed to ApoE deficient mice.* Wildtype and $Tie1^{+/-}$ mice will be analyzed for the onset and progression of atherosclerosis. Floxed Tie1 conditional-knockout mice will be crossed to tamoxifen-inducible endothelial-specific Cre mice on the ApoE background ($Tie1^{flox/flox}$:SCL-ER^{T}-Cre:$ApoE^{-/-}$) and the effect of Tie1 loss on the progression of atherosclerosis will be compared to littermate controls.

Aim 3. *Determine the role of shear stress activated Tie1 signaling on endothelial cell inflammation.* Primary murine aortic endothelial cells will be generated from

Tie1$^{flox/flox}$:SCL-ER^{T}-Cre mice and used in an *in vitro* shear stress culture system with and without the addition of 4-hydroxytamoxifen to determine the effect of Tie1 deletion on eNOS expression and nf-κB activation.