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Gene Regulation in Endothelial Cells

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The vascular endothelium exists in a mechanically active environment that includes both fluid shear stress generated by flowing blood and stretching forces generated by transmural pressures. The magnitude and direction of these forces vary with time and location in the vasculature. In addition to the wide variety of physical forces to which the endothelium must sustain, changing local velocity profiles can alter the delivery and removal rate of species to and from the endothelial surface by fluid convection. The continual presence of physical forces generated by steady or time-dependent flows can regulate endothelial cell gene expression with likely subsequent effects on vessel wall biology. This chapter explores physiological and pathological instances where regulation of endothelial gene expression and protein secretion by hemodynamic forces may control vessel function. Recent work by several laboratories has shown that endothelial cell protein secretion is altered by hemodynamic forces and that these changes likely occur at the genetic level. A summary of research investigating endothelial cell gene expression in the presence of fluid shear stress is shown in Table 4.1.

HEMODYNAMICS, MECHANICS, AND MASS TRANSFER

In arterial vessels, the endothelium is often aligned and elongated in the direction of flow in locations where shear forces are high and predominantly unidirectional (65, 89). Alignment of the endothelium can be quite distinct at the flow divider of arterial bifurcations where early atherosclerotic lesions are seldom found. This elongated morphology can be recreated *in vitro* when endothelial cells are exposed to unidirectional shearing forces greater than about 8 dynes/cm^2 for over 24 hours (13, 20, 54). Alignment of endothelial cells occurs with concomitant changes in cytoskeletal structure (24). Presently, it is not known if the shear-induced changes in the cytoskeleton alter cellular function or if the cytoskeletal changes are an endpoint of the cellular adaptation to shear stress. The morphological changes involve specific changes in actin stress fibers, microtubules, and adhesion plaques, and are dependent on cell type, material substrate, and culture density. Phenotypic changes of endothelial cells observed *in vitro* in response to unidirectional shear stress take place during this slowly evolving elongation of cellular morphology. Differentiating the role of the endothelial cytoskeleton as a receiver of mechanical stimuli and,

TABLE 4.1. Modulation of endothelial gene expression by fluid shear stress

Gene	Effect	Time scale	(Ref.)
	mRNA Levels		
tPA	Large increase at arterial levels	24 hr	(14)
Endothelin	Large decrease at arterial levels	24 hr	(86, 50)
bFGF	No effect	24 hr	(14)
Glyceraldehyde 3-phosphate dehydrogenase	No effect No effect	24 hr 1–4 hr	(14, 86) (37, 38)
IL-1, IL-6	No effect	6–24 hr	(59)
PDGF A chain	Tenfold transient increase at arterial levels	2–4 hr	(37)
PDGF B chain	Two- to threefold transient increase at arterial levels	2–4 hr	(38)
	Transcription site identified	< 6 hr	(73)
c-fos	Large increase at arterial levels	0.5 hr	(39)
	Protein synthesis & secretion		
tPA	Increase at arterial levels	4–24 hr	(15, 14)
Endothelin	Decrease at arterial levels	1–24 hr	(86)
	Decreased at high flow in canine arteriovenous fistula	2 wk	(61)
PAI-1	No effect or slight decrease	1–24 hr	(15)
Fibronectin	Slight decrease	24–48 hr	(32)
IL-1, IL-6	No effect	6–24 hr	(59)
c-fos	Induction and nuclear localization at arterial levels	1 hr	(71)

upon stressing, a generator of secondary messengers is largely unstudied. Nonetheless, these morphological changes provide direct evidence that active biochemical processes are occurring due to the sustained shear forces exerted on the endothelium. To date, the molecular level characterization of mechanotransduction includes the identification of a shear stress-activated whole cell potassium current with a conductance of 150 pS (69) and nonselective cation stretch-activated channels (Ca > K > Na > Cs) with conductances of 40 to 56 pS (53). Stretch-activated channels are not likely to be opened by the comparatively small membrane tensions generated by physiological wall shear stresses.

Quite distinct from fluid shear forces are mechanical strains caused by deformation of vessel wall and endothelial cell structures. Mechanical strains (on the order of 1% to 10%) are predominantly circumferential, and occur with the distension of the vessel due to pressure gradients across the vessel wall. The mechanical coupling of the endothelium to its matrix may not be exactly one to one. Mechanical strains can also alter cellular morphology, forcing cellular elongation in the direction perpendicular to the strain axis (41). During invasive procedures such as bypass surgery or grafting, or balloon angioplasty,

large physical forces are imposed upon the endothelium and underlying tissue structures, and are often correlated with vascular pathologies (8, 85). The signaling pathways and mechanism of mechanochemical transduction may be quite distinct when cells are strained and undergo macroscopic deformation (on the order of microns) compared to signaling events caused by fluid shear forces that generate stresses on cellular structures without rapid macroscopic deformations.

Investigations of the rapid responses of the endothelial cell to the onset of shear stress unexpectedly demonstrated the importance of mass transport phenomena by fluid convection. These situations arise when a rapid reaction, relative to diffusion, alters the local boundary layer concentration of a molecular species. Species that are unreactive or slowly consumed or produced at the endothelial cell surface are expected to have surface concentrations quite similar to the bulk concentration, independent of flow rate. Elevated calcium levels in shear stressed endothelial cells (1) appear to be mediated by adenosine triphosphate (ATP) transport to the purinergic receptors on the cell surface, facilitated by the perfusion media (62, 18, 68). ATP is rapidly degraded by endothelial ectoATPases. *In vivo,* the augmented diffusion and mixing by blood cell rotation near the endothelium would be expected to disrupt or reduce concentration boundary layers. Some researchers, however, have detected small increases of intracellular calcium with the onset of fluid flow using cultured endothelial cells (87, 26) in the absence of ATP in the perfusion media. Endothelial cells *in vivo* may possess the ability to mobilize calcium when exposed to changing shear forces, independent of mass transfer effects or vessel distension—an ability that is possibly lost or variable in culture. Many questions remain and further investigations are required to understand mechanotransduction in endothelial cells exposed to shear forces.

The concentration profile of a reactive species C_i (for example, ATP, endothelial derived relaxing factor (EDRF), or prostacyclin) near endothelial surfaces can be predicted if accurate data exist for the diffusion coefficient (D, cm^2/sec), velocity profiles (v, cm/sec), and consumption or production rate ($\pm R$, moles/cm^2-sec) at the endothelial surface. The reaction rate may be dependent on surface concentrations. The convection-diffusion equations with reaction at the endothelial surface are given below for straight, nontapering cylindrical vessel geometries (equations 1–3) and planar geometries (equations 4–6). In this formulation, the diffusion coefficient near the surface is assumed to be independent of shear rate, otherwise it would be included inside the differential operators. For completeness, the wall shear stresses and flow-pressure drop relations are also given. In these equations, R is the tube radius, $\Delta P/L$ is the pressure gradient, Q is the volumetric flow rate, μ is the fluid viscosity, and r and z are the radial and axial coordinates, respectively. Normally, the axial dispersion term is neglected because it is small relative to radial diffusion and axial convection in most liquid systems. In the parallel-plate geometry, W is the width of the channel, b is the half-gap separation between the parallel plates (2b is the total plate separation) where y ranges from 0 at the midway position between the two plates to $\pm b$ at each plate, and z is the axial coordinate in the direction of flow.

Cylindrical Geometry

velocity profile flow-pressure drop relation (1a,b)

$$v(r) = \frac{R^2}{4\mu}\left(\frac{\Delta P}{L}\right)\left[1 - \left(\frac{r}{R}\right)^2\right] \qquad Q = \frac{\pi R^4}{8\mu}\left(\frac{\Delta P}{L}\right)$$

convection/diffusion equation with boundary conditions (2)

$$\frac{\partial C_i}{\partial t} = \mathcal{D}\left[\frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial C_i}{\partial r}\right) + \frac{\partial^2 C_i}{\partial z^2}\right] - v_z(r)\frac{\partial C_i}{\partial z}$$

$$\left.\frac{\partial C_i}{\partial r}\right|_{r=0} = 0$$

and

$$\mathcal{D}\left.\frac{\partial C_i}{\partial r}\right|_{r=R} = \pm \mathcal{R}(C_i)\,|_{r=R}$$

$C_i(r, z, t = 0)$ and $C_i(r, z = 0, t)$ prescribed.

wall shear stress (3)

$$\tau_w = -\mu\left.\frac{dv(r)}{dr}\right|_{r=R} = \frac{R}{2}\left(\frac{\Delta P}{L}\right) = \frac{4\mu Q}{\pi R^3}$$

Parallel-Plate Geometry

velocity profile flow-pressure drop relation (4a,b)

$$v_z(y) = \frac{b^2}{2\mu}\left(\frac{\Delta P}{L}\right)\left[1 - \left(\frac{y}{b}\right)^2\right] \qquad Q = \left(\frac{2}{3}\right)\frac{Wb^3}{\mu}\left(\frac{\Delta P}{L}\right)$$

convection/diffusion equation with boundary conditions (5)

$$\frac{\partial C_i}{\partial t} = \mathcal{D}\left[\frac{\partial^2 C_i}{\partial y^2} + \frac{\partial^2 C_i}{\partial z^2}\right] - v_z(y)\frac{\partial C_i}{\partial z}$$

$$\left.\frac{\partial C_i}{\partial y}\right|_{y=0} = 0$$

and

$$\mathcal{D}\left.\frac{\partial C_i}{\partial y}\right|_{y=\pm b} = \pm \mathcal{R}(C_i)\,|_{y=\pm b}$$

$C_i(y, z, t = 0)$ and $C_i(y, z = 0, t)$ prescribed.

wall shear stress (6)

$$\tau_w = -\mu \left.\frac{dv(y)}{dy}\right|_{y=\pm b} = \left(\frac{\Delta P}{L}\right) b = \left(\frac{3}{2}\right)\frac{\mu Q}{Wb^2}$$

Linearization of the parabolic velocity profile very near the surface reduces the vessel geometry to a planar system (42). Using this approach, Nollert et al. (68) predicted that step changes from no flow to high flow (wall shear stresses of 4 to 25 dynes/cm^2) cause the surface concentration of ATP to increase from near zero values to steady state values, which approach the bulk concentration of ATP in the perfusion media. This experimental situation has been verified by several laboratories (62, 18, 68) to cause calcium transients mediated by ATP mass transfer phenomena.

Often during *in vitro* experimentation, the issue is raised concerning oxygen limitations. In parallel-plate experiments, the typical use of 1 ml media per cm^2 of monolayer (~100,000 cells/cm^2) is 10 to 20 times the media volume used to grow exponential phase mammalian cells in a suspension culture. Oxygen consumption by cultured, confluent human endothelial cells is expected to be much less than the consumption rate of 0.04 to 0.4 mMole $O_2/10^9$ cells-hr for exponential phase mammalian cells (21). Circulating medium in parallel-plate configurations used in the present research (average residence time in chamber <2 seconds) is well oxygenated [0.2 mM O_2]. Thus, the delivery of oxygen to the monolayer surface exceeds by several orders of magnitude the consumption of oxygen, which is estimated to be less than 9×10^{-10} mMole O_2/cm^2-sec. The concentration of oxygen near the cell surface is greater than 95 percent of the bulk O_2 concentration for wall shear stresses greater than 10^{-3} dynes/cm^2.

In considering the direct effects of fluid shear stress on endothelial gene expression, the role of mass transfer becomes important, since shear stresses are usually generated experimentally by increasing the flow rate. As flows increase, the delivery and removal rates of molecules to and from the cell surface are enhanced. Even at low venous levels of shear stress, mass transfer occurs at rates that are orders of magnitude faster than those found in stationary cultures. Changes in endothelial cell function that occur at arterial stresses, but not at venous or stasis conditions, are likely *direct* stress effects, since

mass transfer effects would be prominent even at venous levels of stress on the order of a few dynes per cm.[2]

Autocrinic mechanisms in endothelial cells present challenging problems in terms of experimental design to evaluate the *direct* effect of shear forces on gene expression. For example, if flow enhances EDRF production (36, 70, 75) via the forces on the plasmalemma with resulting activation of NO synthase, then the direct effect of shear stress is to alter cellular function. However, if EDRF generated by flow is maintained locally at sufficient concentrations, then an autocrinic enhancement of cGMP is impossible—a secondary result of the direct shear effect. Elevated cGMP levels could modulate expression of genes that are responsive to changes in cGMP levels (3, 50). The difficulty in understanding such a phenomenon is that under conditions of high flow (and high shear stress) the removal rate of an autocrinic species from the cell surface may be enhanced. For example, high flows could reduce the local concentration of EDRF, which in turn would enhance the concentration gradient for EDRF diffusion out of the cell. Similar phenomena may be relevant for prostacyclin production, which is induced by shear stress (29, 22) and which can cause enhanced levels of cAMP via an autocrinic mechanism (72). The competition between shear stress induction and flow-dependent removal of an autocrinic mediator can influence the design and evaluation of *in vitro* and vessel perfusion systems for investigations of endothelial gene expression in the presence of fluid shear stress.

PROTEIN SECRETION PATHWAYS

Endothelial cells express a wide variety of membrane receptors and secreted proteins that play an important role in thrombosis, fibrinolysis, inflammatory pathways, hypertension, smooth muscle cell proliferation, and cell adhesion. Protein secretion can involve both constitutive or inducible pathways, and can also be controlled by the rates of mRNA transcription, mRNA degradation, or mRNA translation. Since fluid shear forces cause enhanced arachidonic acid metabolism (29, 22) and endocytosis (12), and morphological elongation (65, 89, 13, 20, 54, 24), changes in secretion rates of proteins are not unexpected.

In our own studies of protein secretion in endothelium exposed to defined levels of laminar fluid shear stress, we used primary human umbilical vein endothelial cells (HUVEC) grown on glass slides (15, 14, 86). Replicate primary, confluent monolayers were maintained in stationary culture or exposed to steady shear stresses of 4, 15, or 25 dynes/cm^2 in individual parallel-plate flow chamber systems with recirculating medium driven by a constant hydrostatic head (Fig. 4.1). Flow chambers were connected under sterile conditions to individual flow systems, each filled with 15 ml of circulating medium. This parallel-plate system offers the benefits of real time microscopic observation of the cells, accurate control of perfusion media pH and oxygen, and circulating volumes (1 ml per cm^2 of monolayer), which are suitable for direct protein measurements by immunoassay. While cone-and-plate systems are ideal for shearing suspensions of blood cells such as platelets, these configurations are difficult to operate under sterile conditions and may potentiate autocrinic

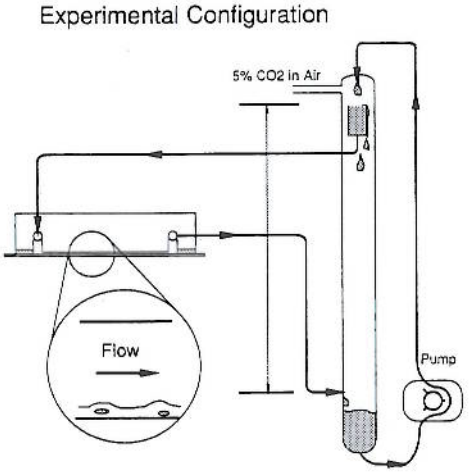

FIGURE 4.1. Schematic of parallel plate flow system.

mechanisms if the fluid to cell volume is lower than about 0.1 ml per cm^2 of monolayer. The cone-and-plate viscometer often prevents (1) microscopic evaluations of the cells during shear exposure, (2) continuous sampling of the fluid above the cells to evaluate protein secretion, and (3) accurate control of pH and oxygen content of the media. In our parallel-plate system, the wall shear stress imposed upon the monolayer was evaluated by solution of the Navier-Stokes equation for laminar flow of a Newtonian liquid. The wall shear stress (τ_w = dynes/cm^2) was calculated according to equation 6 where: flow rate, Q = cm^3/sec; viscosity, μ = 0.01 poise; total plate to plate separation, 2b = 0.02 cm; and width, W = 2.49 cm. The entrance length needed for steady parabolic flow to be established was less than 1 mm (23). The Reynolds numbers for all the flow conditions were less than 50, insuring that the flow was truly laminar with no possibility of turbulence.

During the first 4 to 6 hours after the onset of shear stress, endothelial cells secrete tPA at the same rate as stationary controls (Fig. 4.2). The onset of flow does not induce the acute release of any detectable levels of tPA antigen. Low venous shear stress (4 dynes/cm^2) had no effect on tPA secretion relative to static controls over the entire time course of the 24-hour experiments. After 4 to 6 hours of exposure to arterial shear stresses of 15 and 25 dynes/cm^2, however, the level of tPA in the circulating media of the sheared cells exceeded that of static controls. Increase of tPA in the circulating media was linear with time for over 20 hours, allowing a least-squares fit of the steady state secretion rate. The steady state tPA secretion rates of cells exposed to 15 and 25 dynes/cm^2, normalized to matched, unsheared controls, increased 2.06 ± 0.39 (n = 3) and 3.01 ± 0.53 (n = 3) fold over static cultures, respectively (Table

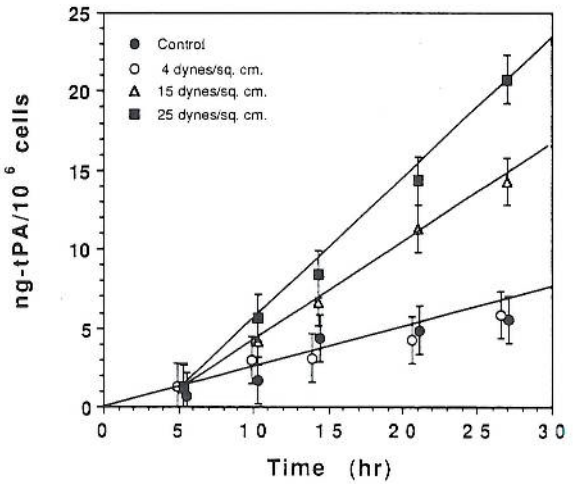

FIGURE 4.2. The secretion of tissue plasminogen activator (tPA) by cultured human endothelial cells exposed to fluid shear stress.

4.2) (15). That venous levels of fluid shear stress had no effect on tPA secretion compared to stationary control would suggest that the greatly enhanced mass transfer at 4 dyne/cm^2 had no effect on the cellular pathways involved in tPA gene regulation and expression. The lag time of several hours prior to enhanced tPA secretion indicates that a transcriptional step was likely needed. Since the intracellular antigen level of tPA of sonicated lysates of stationary cultured cells was very low (<1.0 ng-tPA/10^6 cells) compared to the amount of antigen secreted over the time course of the experiments, the enhanced secretion rate of tPA likely required enhanced synthesis. Thus, the rise of tPA in the

TABLE 4.2. The effect of laminar fluid shear stress on the steady-state tPA secretion rate of cultured human endothelial cells. The average tPA secretion rate of the static controls was 0.168 ± 0.053 ng of tPA per 10^6 cells per hour (n = 3).

	Normalized secretion rate			
Experiment	Static control	4 dynes/cm^2	15 dynes/cm^2	25 dynes/cm^2
1	1.00 ± 0.21	0.92 ± 0.15	2.49 ± 0.39	3.46 ± 0.56
2	1.00 ± 0.07	0.94 ± 0.09	1.95 ± 0.12	3.14 ± 0.08
3	1.00 ± 0.23	0.62 ± 0.13	1.73 ± 0.30	2.42 ± 0.40
Mean[a] ± S.D.	(1.00)	0.83 ± 0.18 (NS)	2.06 ± 0.39 (p<0.015)	3.01 ± 0.53 (p<0.015)

[a]Each mean is the average of three independent experiments involving three separate primary cultures (NS; not significant).

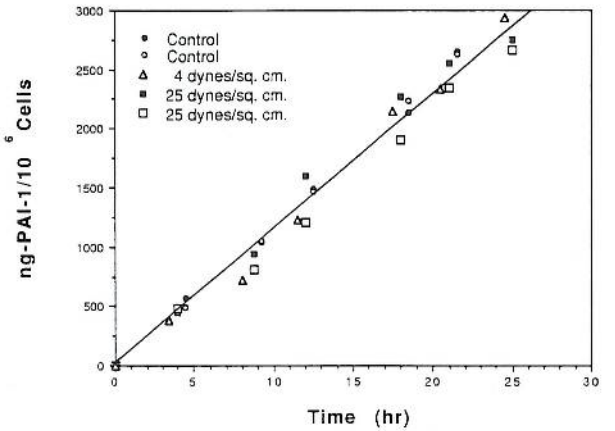

FIGURE 4.3. The secretion of plasminogen activator type 1 (PAI-1) by cultured human endothelial cells exposed to fluid shear stress.

circulating media was not due to release of intracellular stores of tPA due to cell lysis. This is further supported by our observation of a complete blockage of tPA secretion in stationary cultures with cycloheximide (5 μg/ml), suggesting that new protein synthesis was continually required in order to maintain constitutive tPA release. We have found that blockade of cyclooxygenase by low levels of indomethacin (50 μm) had no effect on shear stress induction of tPA secretion. This is consistent with studies indicating that cyclooxygenase activity is not required for tPA secretion by HUVEC (34).

Secretion of uncomplexed PAI-1 antigen into the culture media by stationary cultures or cultures exposed to shear stress was linear with time (Fig. 4.3) but not a function of shear stress. We have found that fluid at venous (4 dynes/cm^2) or arterial (15 to 40 dynes/cm^2) levels of shear stress caused no significant changes in the PAI-1 secretion rate relative to stationary controls (15). The average PAI-1 secretion rate of HUVEC in stationary culture was 53 ± 37 ng-PAI-1/10^6 cells-hr. Secretion of PAI-1 was also unaffected by the presence of indomethacin in stationary or shear stressed cultures.

The vascular endothelium regulates vessel diameter through the release of prostacyclin, endothelial derived relaxing factor, and endothelin (ET). Endothelin is 21 amino acid peptide, which can act as a potent vasoconstrictor. Also, endothelin is a smooth muscle cell mitogen (35, 44), which is synergistic with PDGF (4). The original discovery of ET (91) was accompanied by the observation that fluid shear stress decreased ET production in subcultured porcine endothelial cells (passage 10). In later work, Yoshizumi et al. (93) found that endothelin mRNA levels were higher in porcine aortic endothelial cells (PAEC) exposed to shear stress above 1 dynes/cm^2 but declined at higher levels of 10 dynes/cm^2. They found that endothelin mRNA levels return to basal levels when cells were aligned in the direction of flow. Secretion of endothelin,

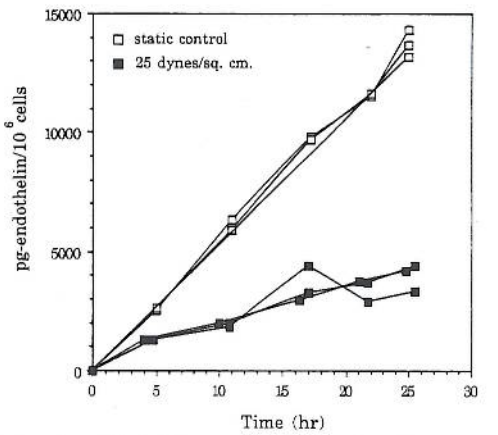

FIGURE 4.4. The secretion of Endothelin type 1 (ET-1) by cultured human endothelial cells exposed to fluid shear stress.

however, was unaffected in PAEC until after nearly 48 hours of exposure to shear stress.

In our studies with HUVEC, we have found a consistent and repeatable suppression of ET expression by arterial levels of fluid shear stress of 25 dynes/cm^2 that is sustained for over 24 hours during stress exposure. A decrease of 60% ± 20% ($n = 6$; $p < 0.01$) in ET peptide release rates was observed within 4 hours after the onset of arterial shear stress of 25 dynes/cm^2 (Fig. 4.4). In human endothelial cells exposed to arterial levels of fluid shear stress, there is simultaneous enhancement of the steady state tPA secretion rate (from 4 to 24 hours) with down regulation of the endothelin steady state secretion rate (Fig. 4.5). During these phenotypic changes we have never observed enhancement of PAI-1 secretion and have seen in some experiments a slight reduction of PAI-1 secretion at arterial shear stresses. Kuchan and Frangos (50) have reported a similar strong and sustained suppression of ET-1 protein secretion when endothelial cells were exposed to arterial flow of 10 dynes/cm^2. Blockade of NO production with L-arginine analogs prevented arterial flows from suppressing ET-1 secretion, suggesting an autocrinic role for NO and cGMP in endothelial cells. Interestingly, they report that low shear stress of 1.8 dynes/cm^2 caused an enhancement of ET-1 secretion over the first six hours of flow.

In a study by Gupte et al. (32), fluid shear stress exposures of 12 hours reduced by over 50% the intracellular content and secretion of fibronectin by HUVEC. Reductions of fibronectin content in the endothelial cell matrix were also observed. These reductions were seen after 48 hours of exposure to shear stress.

Very little data is available on protein secretion in endothelial cells subjected to pulsatile flows. Pulsatile fluid flow (±10% amplitude; 1 Hz) increases PGI_2 production even more than the enhancement by equivalent mean values

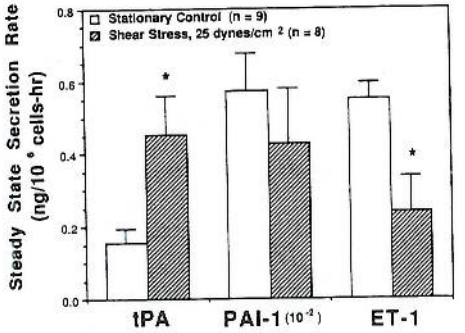

FIGURE 4.5. Steady state secretion rates for tPA, PAI-1, and ET-1 by cultured human endothelial cells exposed to 25 dynes/cm² of fluid shear stress for 24 hours (*$p < 0.001$). Note that the secretion of PAI-1 is over one hundredfold greater than that for tPA and ET-1.

of steady shear stress (22). Davies et al. (12) found that 1 Hz oscillations in flow had no effect on fluid phase endocytosis, while step changes in flow every 15 minutes enhanced endocytosis. In our own studies, we have found that 1 Hz pulsatile shear stress had the same effect as equivalent mean levels of steady shear stress on the secretion of tPA, PAI-1, and endothelin type 1.

GENE EXPRESSION

The time dynamics for changes in protein secretion to occur with endothelial cells subjected to fluid shear forces are suggestive of changes in mRNA transcript levels. A difficult limitation when studying gene expression in human endothelial cells is the limited number of cells available for RNA isolation. Research of changes in mRNA levels in cells subjected to shear stress has been conducted in several laboratories. These studies have used direct Northern blotting and coupled-reverse transcription/polymerase chain reaction (RT/PCR) methods to evaluate mRNA levels.

In our own studies, we have used replicate HUVEC monolayers either maintained in stationary culture or exposed to shear stress at 25 dynes/cm² in individual flow systems. In each experiment, the quantity of total cellular RNA isolated from stationary and shear-stressed HUVEC cultures was not significantly different (average variation less than 10%). To study mRNA transcript levels in limited numbers of primary human umbilical vein endothelial cells without the use of serial passage or growth factor-stimulated expansion of cell numbers in culture, we performed reverse transcription of mRNA followed by a polymerase chain reaction (PCR) as previously described (14, 86).

We have found that the tPA mRNA level was elevated in endothelial cells exposed to arterial shear stress for 24 hours (Fig. 4.6, upper left). In contrast,

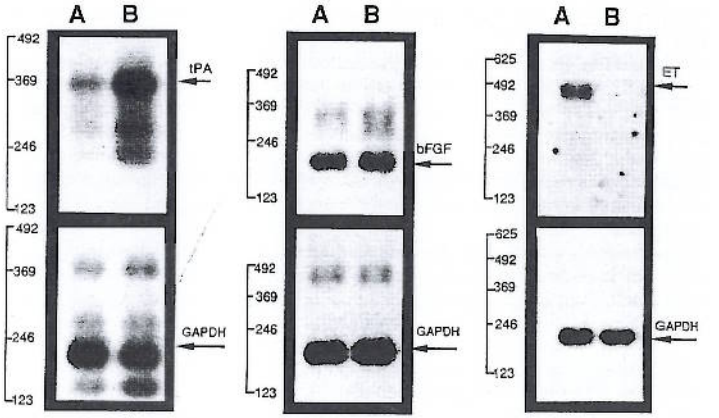

FIGURE 4.6. Messenger RNA levels (as indicated by amount of RT-PCR product) in cells maintained in stationary culture (Lanes A) or exposed to steady laminar fluid shear stress of 25 dynes/cm² (Lanes B) for 24 hours. Expected PCR product sizes are indicated by arrow. The GAPDH mRNA was coreverse transcribed and coamplified in each PCR reaction tube to serve as an internal amplification control for tPA (*left*), bFGF (*middle*), and ET-1 (*right*). See text for details.

when the same Southern blots were stripped and reprobed for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification product, the transcript level of GAPDH was constant and independent of shear stress (Fig. 4.6, lower left). When this RNA from control and stressed monolayers was used in a similar coamplification experiment with GAPDH to study the effect of shear stress on levels for bFGF mRNA, no large increase in transcript levels such as that seen for tPA was noted for basic fibroblast growth factor (bFGF) (Fig. 4.6, middle). Endothelin-1 mRNA levels were sharply reduced in the endothelial cells exposed to 25 dynes/cm² for 24 hours (Fig. 4.6, right). Again, no correspondingly sharp decrease was observed in signal strength for GAPDH product, indicating the uniformity of the coamplification reactions for bFGF and endothelin. The tPA mRNA level was found to be manyfold higher in cells exposed to shear stress compared to stationary controls indicating that the flow response for ET is opposite that of tPA.

In studies of gene regulation in the first few hours after the onset of fluid flow, Hsieh et al. (37) found a transient enhancement of platelet-derived growth factor (PDGF) A and B chain mRNAs, which peaked at about 2 hours exposure to shear stress of 16 dynes/cm². At 4 hours exposure, the mRNA levels returned to resting levels. No measurements of PDGF homo and heterodimers were conducted. Using inhibitors of protein kinase C such as H7 and staurosporine, Hsieh et al. (38) found a strong inhibition of shear-enhanced PDGF A and B mRNA levels. Inhibitors of protein kinase A, cyclooxygenase and G proteins had only partial inhibitory action of shear-enhanced PDGF mRNA levels.

In these studies, the GAPDH mRNA levels were not effected by shear exposures.

In similar work to study the induction of PDGF mRNA transcription in endothelial cells exposed to shear stress, Resnick et al. (73) have used transient expression of PDGF B promoter/CAT (chloramphenicol acetyltransferase) reporter gene constructs with various deletions in the promoter region. In this study, they have identified a novel shear-stress responsive element (GAGACC) that is required for shear induction of the PDGF B promoter construct. In these studies, no demonstration of changes in PDGF B production has been conducted.

In a recent study of the effect of flow on transcriptional activators, Hsieh et al. (39) have found that steady and pulsatile arterial shear stress can induce c-fos mRNA to peak levels within 30 minutes, which then return to baseline within 1 hour. The levels of c-jun mRNA and c-myc mRNA were only moderately enhanced under these conditions. The protein kinase C inhibitor H7 as well as the intracellular calcium chelator Quin 2 blocked the induction of c-fos mRNA by shear stress. Inhibition of cAMP- and cGMP-dependent kinases with HA1004 had no effect on c-fos induction by shear stress.

In our own work, we have used immunocytochemistry for c-fos protein and quantitative fluorescence video microscopy to investigate the effect of fluid shear stress on c-fos levels in human endothelial cells (71). The nuclear-localized c-fos protein level was over five fold higher in the cells exposed to arterial levels of shear stress as compared to the quiescent cells maintained in stationary culture (Fig. 4.7). In contrast, the cells exposed to low levels of shear stress (4 dynes/cm^2) showed diffuse but slightly elevated levels of c-fos without preferential nuclear localization. The protein kinase C inhibitor, H7 (10 μM), significantly attenuated the induction of c-fos by 50% in cells exposed to arterial shear stress for 1 hour. The marked translocation of newly synthesized c-fos protein into the nucleus suggested that the activity of cytoplasm retention factors and the DNA-binding affinity of the AP-1 complex changed in HUVEC exposed to flow.

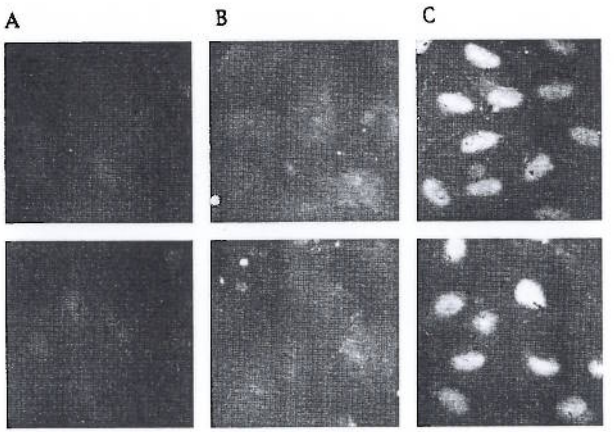

FIGURE 4.7. Localization and immunofluorescence intensity of c-fos protein in human umbilical vein endothelial cells maintained in stationary culture (A) or exposed to fluid shear stress of 4 (B) or 25 dynes/cm^2 (C) for 1 hour. The perfusion media was HEPES-buffered PBS with 1% BSA, containing no extracellular ATP. Two representative views of each monolayer are shown.

ENDOTHELIAL CELL PHENOTYPES

Through expressed metabolites, the endothelium regulates activation and inhibition of blood coagulation, vasoconstriction and dilation, and smooth muscle cell inhibition and proliferation. With respect to fibrinolysis, the endothelium is the principal source of tPA found in the bloodstream. Production of tPA and PAI-1 by endothelial cells is known to be enhanced by cytokines. Coordinated induction of both tPA and PAI-1 by thrombin or basic fibroblast growth factor (bFGF) is in contrast with the induction of tPA alone by histamine, activated protein C, and phorbol ester, or the induction of PAI-1 alone by lipopolysaccharide (LPS), transforming growth factor-β (TGFβ), interleukin-1 (IL-1), or tumor necrosis factor-α (TNFα) (78, 79, 82, 84, 56, 19).

Cultured human endothelial cells secrete more tPA when exposed to arterial levels of shear stress (> 15 dynes/cm^2) while PAI-1 secretion remains unaffected by shear stress over the physiological range. The PAI-1 gene is a very sensitive indicator of cytokine activator of HUVEC (84, 19), and the lack of shear stress induction of PAI-1 would suggest that cytokines are not playing an autocrinic role in the endothelial response to shear stress. This is consistent with reports that fluid shear stress does not increase IL-1 or IL-6 mRNA or IL-1 or IL-6 protein expression (59). The shear response is also distinct from thrombin, basic fibroblast growth factor, or LPS stimulation of endothelial cells, all of which increase PAI-1 secretion.

During acute exercise, mental stress, venous occlusion, and surgery, the rapid release of tPA into the plasma, presumably by the endothelium, has been well documented. The rapid time scale of these *in vivo* phenomena would argue against a role for changes in protein synthesis. There exists only a single report of rapid, induced release of stored tPA by cultured endothelial cells, which was detectable by a sensitive activity assay (2). In cultured endothelial cells, the majority of tPA synthesized is secreted, indicating that intracellular stores of tPA are small and that an important regulator of tPA production is the level of tPA mRNA. Stimulation of endothelial tPA secretion by agents such as butyrate has previously been shown to correlate with large increases in tPA mRNA levels (46). In the case of the HUVEC response to shear stress, the large change in tPA mRNA levels with only threefold enhancement of tPA secretion suggests that an additional level of posttranscriptional regulation of tPA protein production may be present.

In contrast to enhancement of tPA expression by arterial levels of shear stress, expression of endothelin is suppressed by these exposures. The endothelin-1 (ET-1) gene contains: 5 exons, a CAAT box and TATAA box, three putative AP-1 sites, a putative NF-1 site, several putative acute phase regulatory

elements, and several AUUA sequences in the 3′ untranslated region that may be involved in mRNA degradation (40). The ET mRNA is noted for its short half life (90). Study of the endothelin gene *in vitro* is complicated by the fact that changes in mRNA levels may not produce changes in protein secretion. For example, the severalfold transient induction of the ET-1 mRNA by phorbol ester, which peaks at 20 minutes, is followed by a sharp down regulation of the gene at 2 hours (90). The transient increase of ET-1 mRNA by phorbol ester is not accompanied by enhancement in ET secretion (9, 58). Often, upon stimulation of the endothelial cell by an agonist, ET expression is transiently increased and then strongly down-regulated relative to basal levels. The poor stability of ET mRNA likely has an important role in this down regulation, since ET mRNA levels have been shown to drop to very low levels after stimulation by agents such as phorbol ester (90) and TNF-α (57). In contrast, IL-1 caused up to a fivefold enhancement of ET-1 mRNA levels with a 1.6-fold increase in ET-1 protein secretion without downregulation of the gene over 48 hours (92).

Under conditions of chronic exposure to high shear stress, the enhancement of cAMP levels via a PGI_2 mediated adenylate cyclase activity (72) and the possible enhancement of cGMP via EDRF (3, 50) could alter endothelial phenotype under flow. It is known that elevated cAMP levels under conditions of protein kinase C (PKC) activation lead to large induction of tPA with slight reduction of PAI-1 expression (55). Elevation of the intracellular cAMP level alone using forskolin, cAMP analogs, phosphodiesterase inhibitors (or combinations of these agents) does not stimulate secretion of tPA by HUVEC but rather slightly attenuates production over a 20-hour period (55). Interestingly, phorbol ester causes a very large induction of tPA with only small effects on PAI-1 secretion (55) despite the presence of two activator protein 1 (AP-1) elements in the PAI-1 promoter, which are likely active *in vivo* (81). LPS, however, is an important inducer of the PAI-1 gene both *in vitro* and *in vivo*. In culture, basal endothelial secretion of PAI-1 appears to be unusually high in relation to the secretion level of tPA and the low concentration of PAI-1 found in plasma. Both human umbilical vein and bovine aortic endothelial cells produce substantial quantities of PAI-1, which accounts for about 1% to 10% of total secreted protein.

There are several lines of evidence suggesting a possible role for PKC activation in phenotypic changes in response to shear stress. Enhanced PGI_2 production may result from enhanced availability of arachidonic acid via the activity phospholipase A_2 and/or phospholipase C (29, 22). Given the enhanced incorporation of arachidonic acid into phosphoinositol and diacylglycerol in HUVEC exposed to shear stress (67), some activation of protein kinase C may occur. In endothelial cells exposed to flow, the enhanced generation of inositol trisphosphate (IP_3) should lead to increased levels of the PKC activator diacylglycerol (66). Activation of protein kinase C is known to enhance tPA gene expression (31) with little effect on PAI-1 production (55) while ultimately down-regulating ET secretion (90, 9). Stimulation of tPA production by diacylglycerol analogs can be blocked by PKC inhibitors like sphingosine or H7 (31). The inhibitors H7 and straurospaurin have been shown to block shear enhancement of PDGF A and B mRNA after 2-hour shear exposures (38). In our

own observations, we have seen H7 block shear stress-enhanced secretion of tPA. However, H7 has many toxic and nonspecific effects (94) that prevent mechanistic evaluations particularly with long term exposures to these agents. PKC inhibitors cause significant disruption of the cytoskeleton, which in turn alters cell morphology under conditions of flow.

It is known that PKC activation can lead to the induction of the proto-oncogenes, c-fos and c-jun (7). One possible regulator of the tPA gene is the c-fos/c-jun complex (AP-1). This complex is rapidly and transiently induced at 15 to 30 minutes after phorbol ester stimulation and returns to basal levels in many cell types within a few hours. Stretching (5%–20% strain) of cultured rat cardiac myocytes caused stimulated expression of c-fos within 15 minutes (45). These workers found that the level of c-fos mRNA peaked at 30 minutes and returned to undetectable levels at 240 minutes. These two proteins bind each other and form a transcriptional activator, which can bind AP-1 promoter sites [5′-TGAGTCAG-3′] of various genes (6, 47). The activation of PKC and concomitant induction of c-fos/c-jun may be a link between signal transduction events in cells exposed to fluid flow and slower changes in genetic expression, which occur over several hours (Table 4.1). This scenario of PKC activation by shear stress is consistent with most experimental observations; however, the role of calcium in sheared endothelial cells remains unclear.

Many studies have shown that low shear stress zones in arteries (with reversing flows) are more prone to develop atherosclerotic lesions, intimal hyperplasia, and enhanced thrombogenicity when compared to vascular regions with unidirectional, high shear flow (for review, see ref. 27). Studies concerning the role of hemodynamic forces in atherogenesis evolved from the recognition that repeated endothelial cell injury may initiate plaque formation (74). It is important to note that turbulent flow is not found under normal physiological conditions, occurring only in some prosthetic valves and vessels with highly diseased stenoses (28).

Examining human carotid bifurcations at autopsy, Zarins et al. (95) found that early lesions were located in regions of flow separations, low shear stress, and complex secondary flows. The outer wall of the carotid sinus (with separating flow and low shear stress) had the most plaques. Few lesions were found near the flow divider where shear stress was high and the flow was primarily axially oriented. Sakata and Takebayashi (76) also found that lesions were localized to the outer wall of the carotid artery. In a similar study, Grottum found lesions in the human left coronary artery bifurcation preferentially on the outer wall where stresses were lowest (30). In human cerebral arteries, the incidence of arteriosclerotic lesions is also higher on the outer walls of the daughter vessels (77). Work by Friedman et al. (25) with pulsatile flow in human aortic bifurcations demonstrated that intimal thickening was inversely related to the magnitude of the shear stress. Vein grafts also experience intimal hyperplasia associated with low or altered flow (17, 16, 64).

Using measurements from adult human angiograms, Ku et al. (48, 49) developed a scaled plexiglass bifurcation model of the carotid artery. With this model bifurcation, Ku et al. correlated intimal thickening (in 12 autopsy specimens) to the outer wall of the proximal internal carotid with low shear stress (mean shear stress = -0.5 dynes/cm^2) and a high degree of flow reversal (in-

stantaneous shear stress = −7 to 4 dynes/cm^2). The inner walls of the internal carotid (mean shear stress = 17 to 26 dynes/cm^2; no flow reversal) had little intimal thickening. Regions of the vessels downstream of the bifurcation had unidirectional flow, high mean shear stress (14 to 45 dynes/cm^2), and no intimal thickening.

Flow-induced vasodilation is often a rapid response of vessels to changing flow and does not represent changes in gene expression. Other observations, however, have focused on vessel tone in relation to chronic changes in blood flow. In a dog model, Dobrin et al. (17) showed that femoral vein grafts dilate to maximum diameter within 4 weeks. Zarins et al. (96) found that arteriovenous fistulas between the right iliac artery and vein (with left iliac artery as control) in cholesterol-fed cynomolgus monkeys had a tenfold greater blood flow compared to the left artery. The two arteries, however, had the same shear stress (15 dynes/cm^2) due to a twofold increase in lumen diameter of the right iliac artery from flow-induced vasodilation. Both vessels had equal amounts of plaque formation after 6 months, indicating that increased blood flow did not enhance atherogenesis. Interestingly, a 70% reduction of blood flow can lead to reduction of vessel diameter by 21% as measured in rabbits after 2 weeks with ligated carotid arteries (52). Also, slight intimal thickening was also observed in these rabbit carotid arteries after 2 weeks of reduced flow.

The time scale of these responses is suggestive of phenotypic changes that might occur in the vessel. A molecular mechanism to explain these observations is suggested in part by shear control of endothelin secretion in cultured human endothelial cells exposed to arterial shear stress. In situations of chronic high flow, endothelin expression may be suppressed allowing dilatory mediators to prevail. In situations of chronic low flow, the endothelin gene is not regulated and high expression of endothelin could lead to vasoconstriction. It may be possible that flow control of endothelin expression has a role in other situations such as angiogenesis and vessel development (51).

Recently, Miller et al. (61) have reported that chronic increases in blood flow (1049 ml/min) though canine femoral arteries with arteriovenous fistulas caused a sharp decrease of nearly 75% in the amount of endothelin antigen extractable from the vessel wall compared to sham operated arteries with flow of 116 ml/min. This reduction of tissue content of ET by flow was seen on a per weight basis and a per surface area basis. The endothelin content measurement in the vessels did not differentiate endothelial or smooth muscle cell production of the vessels. In this model, it could be possible that chronic high flow caused: (1) enhanced ET secretion with concomitant washout of the peptide from the vessel wall thus reducing net content, or conversely (2) suppression of ET expression by the cells of the vessel wall. Since circulating levels of ET are so low and ET-receptor binding so strong, the washout rate at normal arterial flows is limited by diffusional processes in the vessel wall. This diffusion rate would not be affected by flow unless the endothelial permeability changed because of the high flow generated with the arteriovenous fistula. This *in vivo* observation, in conjunction with the observation of vessel constriction during chronic low flow (52), provides perhaps the first correlation between *in vitro* studies of endothelin expression conducted with human endothelial cells and animals models in dog and rabbit.

Blood vessel walls exposed to high flow may have enhanced PGI_2 production (29, 22) and enhanced fibrinolytic capacity (15, 14), thus increasing the resistance of high shear zones to fibrin deposition on the vessel wall and platelet-depedent and platelet-independent smooth muscle cell proliferation. Our finding that arterial levels of shear stress suppress endothelin production suggests an additional mechanism whereby reductions in intimal thickening might arise from direct local suppression of endothelial cell expression of the smooth muscle cell mitogen, endothelin. Also, in unidirectional, steady arterial flows, the enhanced production of tPA may lead to increased plasmin-mediated conversion of latent TGFβ to active TGFβ (80) with subsequent attenuation of bFGF actions, a presumably beneficial effect.

Attenuated tPA production in low shear stress regions and subsequently reduced fibrinolysis at the endothelial surface may contribute to atherosclerotic plaque development (10). The strongest link between predisposition to atherosclerosis and reduced fibrinolysis is that high serum levels of lipoprotein (a) [LP(a)] are a risk factor for atherosclerosis (11). LP(a) may interfere with plasminogen activation on the endothelial cell surface, thus hindering the fibrinolytic pathway (60, 33). Fibrin causes disorganization of endothelial monolayers (83). Fragment D increases endothelial permeability to albumin; fibrinopeptide B has been shown to be chemotatic for macrophages in early lesions (88). Deficient fibrinolysis may lead to fibrin deposition and enhanced fibrin incorporation into developing atherosclerotic lesions. In zones of high shear stress in the cardiovasculature, enhanced expression of tPA may protect the vessel wall from shear-induced platelet aggregation (63) with thrombin generation and subsequent fibrin deposition.

It has been reported that steady laminar shear stress caused a transient increase in PDGF A and PDGF B chain mRNA, which peaked at 2 hours and returned to baseline levels by 4 hours (37, 38, 73). Nothing is known about PDGF protein secretion by endothelial cells maintained in a flow regime. A study of reendothelized PTFE grafts in baboons suggests, however, that high flow for 3 months is inversely correlated with wall thickness and intimal hyperplasia (43). When a high shear flow was abruptly decreased to a low shear flow, rapid neointimal thickening occurred. These researchers found that the SMC number was increased in grafts exposed to low flow while the fraction of matrix of the intima was unchanged, and implicated PDGF-AA in the vessel wall to mediate the phenomena. Conversely, in vein grafts subjected to chronic high flow of the arterial system, thickening does occur over time (97). In these situations, as distinct from PTFE grafts, distension of the vessel wall may also contribute to the response of the vessel. These *in vivo* studies suggest that hemodynamic shear stress induction of the PDGF A and B gene may not always be a straightforward conclusion.

Transient flows such as pulsatile, reversing flows, or pulsatile flow past a stenosis could possibly provide the continual stimulus for elevated PDGF mRNA levels. Although HUVEC express mRNA for PDGF-A chain and PDGF-B chain and synthesize a PDGF-like protein, PDGF has little autocrine activity, since normal endothelial cells lack the PDGF receptor. The finding that cultured endothelial cells respond to the onset of fluid shear stress with enhancement of PDGF mRNA may reflect more about the step change in flow

and less about the magnitude of shear stress exposure. The work of Kohler et al. (43) with reendothelialized PTFE grafts indicates that high flow reduces intimal hyperplasia, which is the opposite of that predicted from the *in vitro* models (37, 38, 73). This finding would indicate that the evaluation of chronic low and high flow states on vessel wall biology may not be accurately predicted by studies of the transient response of PDGF mRNA levels in endothelial cells exposed to the onset of fluid flow. It is unlikely that such a potent mitogen as PDGF would be used by the vessel as a mediator of vasodilation.

Over the last decade, many studies at the cellular and molecular level have helped to characterize endothelial response to the drag forces generated by flowing blood. It is likely that several intracellular signals occur to produce the wide variety of mechanically-induced changes in gene expression seen when endothelial cells are subjected to flow. Some similarities exist between the response to shear stress and the response to stretching forces due to vessel distension. For example, both stretching and shearing forces cause enhanced prostacyclin (PGI_2) production in cultured endothelial cells (29, 22, 5). However, important distinctions can be made regarding the nature of physical forces in substrate-stretching models (1%–10% strain) and fluid shear systems (1–30 dynes/cm^2). The endothelial response to these different forces displays some heterogeneity. While substrate-stretching has been reported to produce calcium mobilization and enhanced endothelin production (5), fluid shear stress has little effect on intracellular calcium and suppresses endothelin production (86). As molecular and biophysical approaches are applied to explore endothelial responses to physical forces, the data will hopefully converge to provide fundamental insights. These insights must bridge *in vitro* models, animal studies, and clinical observations. In time, the use of gene therapy, antihyperplasia agents, or surgery will gain from this detailed understanding of vascular disease processes that are known to be localized by hemodynamics. In fact, predicting the local secretion rates of gene products from transfected endothelial cells will require a knowledge of how the local blood flow modulates gene expression in these cells.

ACKNOWLEDGMENTS

The authors would like to thank the many clinicians, scientists, and engineers who have made significant contributions to this research. In particular, the authors wish to acknowledge the generous help and outstanding contributions of Drs. Suzanne G. Eskin and John B. Sharefkin.

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5

Flow Effects on Endothelial Cell Signal Transduction, Function, and Mediator Release

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The circulation in animals has evolved to reduce the diffusional distance between the nutrient supply and the cells constituting the organism. Depending on environmental changes and the activity performed by the animal, the metabolic demand by the different tissues can vary significantly. For a long time, it was generally accepted that there were two different control mechanisms for controlling blood flow within the circulation in animals: a systemic control by the central nervous system via the sympathetic neuronal network and by hormones from the adrenal gland, and a local control mediated by the metabolism of the organs. The role of flow as a potential factor for local regulation of vascular function has been recognized more recently. There is now considerable evidence that the size of blood vessels and vascular tone are dependent on the local level of wall shear stress in the vasculature, and that endothelial cells mediate the response of vessels to changes in flow conditions (74, 89, 98, 91, 125, 149, 150, 173, 183). In other words, endothelial cells, which are in direct contact with the flowing blood, act as flow sensors and generate signals to trigger the appropriate response by the vessels. Given the large amount of data showing that various agonists stimulate endothelial cells to secrete various vasoactive and mitogenic substances (for review, see [57]), it appears likely that flow-induced effects on vessel size and tone, which are generally effected by the underlying smooth muscle cells, may also be mediated by substances released by the endothelium.

In this chapter, we summarize the results of various investigators that have reported the effects of flow on endothelial cell mediator production. Most of this work has been performed with cultured endothelial cells from a variety of animal sources. In the first sections, the effect of flow on each mediator studied is analyzed; the emphasis is placed on the biochemical pathways involved in these responses. A section on the effect of flow on second messengers has been included where the early events in flow signal transduction are discussed. Finally, we conclude by summarizing this information in the context of the role of these mediators in the control of vascular tone, diameter, and permeability.