Check for updates

Endothelial Expression of Thrombomodulin Is Reversibly Regulated by Fluid Shear Stress

Adel M. Malek, Robert Jackman, Robert D. Rosenberg, Seigo Izumo

Abstract The vascular endothelium, by virtue of its position at the interface between blood and the vessel wall, is known to play a critical role in the control of thrombosis and fibrinolysis. Thrombomodulin (TM) is a surface receptor that binds thrombin and is a potent activator of the protein C anticoagulant pathway. Although TM expression is known to be regulated by various cytokines, little is known about its response to ever-present biomechanical stimuli. We have explored the role of fluid shear stress, imparted on the luminal surface of the endothelial cell as a result of blood flow, on the expression of TM mRNA and protein in both bovine aortic endothelial (BAE) and bovine smooth muscle (BSM) cells in an in vitro system. We report in the present study that TM expression is regulated by flow. Subjecting BAE cells to fluid shear stress in the physiological range of magnitude of 15 (moderate shear stress) and 36 (elevated shear stress) dynes/cm^2 resulted in a mild transient increase followed by a significant decrease in TM mRNA to 37% and 16% of its resting level, respectively, by 9 hours after the onset of flow. In contrast, shear stress at the low magnitude of 4 dynes/cm^2 did not affect TM mRNA levels. The sensitivity of TM mRNA expression by flow was found to be specific to endothelium, since it was not observed in BSM cells exposed to steady laminar shear stress of 15 dynes/cm^2. Furthermore, unlike BAE cells, BSM cells did not exhibit altered cell shape nor align in the direction of flow after 24 hours of shear stress at 15 dynes/cm^2. In BAE cells, TM protein assessed by Western blot analysis also showed a decrease to 33% after 36 hours of laminar shear stress of 15 dynes/cm^2. The downregulation of TM mRNA in response to shear was found to recover completely to its static level within 6 hours after the cessation of the mechanical stimulus. Furthermore, the process was entirely reversible and without hysteresis, since further shear stimulus after recovery yielded the same behavior. The dynamic character of the shear was varied, and it was found that steady laminar, turbulent, and pulsatile shear stress with a mean magnitude of 15 dynes/cm^2 all resulted in a similar decrease in TM mRNA. Expression of tissue-type plasminogen activator (TPA) mRNA in the same BAE cells, previously reported to be affected by flow in human umbilical vein endothelial cells, was found to be increased 3-fold by 15 dynes/cm^2 and 22-fold by 36 dynes/cm^2 at 9 hours. The reciprocal behavior of TM and TPA to shear stress suggests a switch in endothelial phenotype from a predominantly antithrombotic state under static conditions, dominated by TM expression, to a fibrinolytic state governed by TPA under flow conditions, particularly under elevated shear stress (36 dynes/cm^2). These findings suggest that TM may be playing a localized protective role against thrombosis in regions of stasis and low flow and identify flow as a novel regulator of endothelial TM expression. (***Circ Res.* 1994;74:852-860.**)

Key Words • mechanical forces • gene regulation • thrombosis

Downloaded from http://ahajournals.org by on November 15, 2026

The endothelial cell serves as a functional barrier between blood and the vessel wall and plays a critical role in the control of thrombosis and fibrinolysis in the blood vessel by a number of distinct mechanisms.[1] These regulatory processes include both negative and positive effects at distinct levels, including platelet activation by platelet-activating factor,[2] inactivation by prostacyclin (PGI_2)[3] and endothelial cell–derived relaxing factor (EDRF),[4,5] antithrombin III–dependent neutralization of the multiple serine proteases of the coagulation cascade by heparin-containing proteoglycans on the endothelial surface,[1] and fibrinolysis and its inhibition by tissue-type plasminogen activator (TPA)[6,7] and its inhibitor plasminogen activator inhibitor (PAI-1),[8] respectively.

Of the known mechanisms that serve to negatively control the natural coagulation tendency of blood is the thrombomodulin (TM)–protein C pathway.[9] TM is an integral membrane glycoprotein expressed on the surface of the endothelial cell that functions as a receptor with a high affinity for thrombin and forms with the latter a 1:1 stoichiometric complex that serves as a potent catalyst of the thrombin-induced activation of protein C. Activated protein C complexes to protein S, and together they catalyze the inactivation of factors Va and VIIIa, thereby serving to inhibit thrombin formation.[9] The relative importance of the protein C system is underscored by increased thromboembolic disorders in individuals with protein C or S deficiencies[10] and by the ability of exogenous TM to counteract, and anti-TM antibodies to potentiate, thrombin-induced thromboembolism in mice.[11] Activated protein C has been demonstrated to protect from gram-negative sepsis in a baboon model.[12] Conway et al[13] have also implicated TM in the internalization and clearance of extracellular thrombin. Soluble human recombinant TM has been recently demonstrated to antagonize thrombin receptor–mediated inositol phosphate generation, PGI_2 release, and permeability increase in human umbilical vein endothelial (HUVE) cell monolayers, suggesting a role for TM in modulating endothelial cell activation by thrombin.[14]

In addition to its multifunctional regulatory role, the endothelial cell is constantly exposed to blood flow and

Received July 20, 1993; accepted December 30, 1994.

From the Harvard Medical School–Massachusetts Institute of Technology Division of Health Sciences and Technology (A.M.M., R.D.R., S.I.) and the Molecular Medicine Division (R.D.R., S.I.) and the Department of Pathology (R.J.), Beth Israel Hospital, Boston, Mass.

This manuscript was sent to Leslie A. Leinwand, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Reprint requests to Seigo Izumo, MD, Division of Cardiology, University of Michigan Medical Center, Ann Arbor, MI 48109.

the resulting shear stress, ie, the frictional force per unit area acting tangentially on the endothelial surface in the direction of flow. Shear stress has been shown to be a central regulator of blood vessel structure based on principles of optimality[15-17] by a feedback mechanism that involves the endothelial cell as one of its limbs.[16] Experiments have shown that blood vessels dilate[18] or constrict[19] structurally in response to increases and decreases in blood flow, respectively, so as to maintain shear stress magnitude at ≈15 dynes/cm^2 by a mechanism that is endothelium dependent.[19] Furthermore, areas of the vascular system exposed to fluid shear stress of low magnitude (4 dynes/cm^2) and of time-varying character have been associated with a higher incidence of intimal hyperplasia and atherosclerotic change,[20] suggesting for mechanical shear stress a possible role in the pathophysiology of vascular disease. The endothelial cell is now known to respond functionally and phenotypically to its external mechanical environment (for recent review, see Reference 21); endothelium is characterized by a shear stress–sensitive outward potassium current[22] and responds promptly to shear stress with a transient increase in $[Ca^{2+}]_i$[23-25] and a decrease in pH_i.[26]

Experiments have demonstrated that the coagulation-controlling properties of the endothelial cell are regulated by hemodynamic forces: fluid shear stress induces significant increases in PGI_2 release in HUVE cells,[27,28] and shear stress at the high magnitude of 25 dynes/cm^2, but not at the low magnitude of 4 dynes/cm^2, increases TPA secretion and mRNA levels,[29,30] which, through activation of plasmin, play a key role in fibrinolysis.[7,8] In the same cells, PAI-1 expression was not found to be altered by shear.[29] In addition, numerous reports have documented increases in EDRF release[31,32] with blood flow increases in the short term; recently, shear stress has been shown to chronically increase the expression of nitric oxide synthase.[33] These findings may have implications for the control of hemostasis, since both EDRF and PGI_2 inhibit platelet aggregation.[4]

TM expression has been reported to increase in response to cAMP, retinoic acid, and thrombin,[34-36] and TM surface activity has been reported to decrease in response to endotoxin, interleukin-1, tumor necrosis factor-α,[37] and the thrombogenic agent homocysteine.[38] However, it is not clear if or how mechanical forces resulting from blood flow may regulate TM levels. The hemodynamic stimulus is likely to be an important physiological factor, since vascular endothelium is constantly exposed to flow and the resulting fluid shear stress.[39]

Accordingly, using a previously described in vitro cone-plate viscometer apparatus,[40,41] we have explored the role of well-controlled fluid shear stress in different dynamic regimens on the expression of TM mRNA and TM protein in bovine aortic endothelial (BAE) and bovine smooth muscle (BSM) cells.

Materials and Methods

Cell Culture

BAE cells were harvested from descending thoracic aortas obtained from the local abattoir by collagenase digestion and were used between passages 6 and 15, during which no change in responsiveness was observed. The cells were grown in DME medium (GIBCO) supplemented with 10% calf serum, 4 mmol/L L-glutamine, 25 mmol/L HEPES (pH 7.4), 10 U/mL penicillin, and 10 μg/mL streptomycin at 37°C and 5% CO_2 in a humidified incubator. BSM cells, a gift from Dr Gerald A. Soff, were prepared by enzyme dispersion according to previously published methods[42] and grown in DME supplemented as above and containing 20% fetal calf serum until confluent.

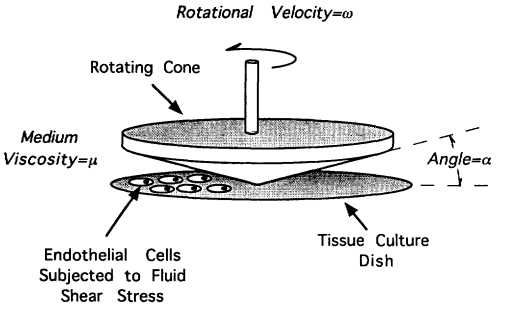


FIG 1. Diagram of cone-plate viscometer apparatus used in this study to apply fluid shear stress to monolayers of adherent cells grown in tissue culture plate. The device consists of a cone of angle α in contact at its tip with the center of a standard 10- or 15-cm tissue culture plate on which are grown cell monolayers. The cone rotates at $\omega 2\pi$ radians/s and imparts via the viscosity of the medium a shear stress acting in the direction of the flow on the surface of the cell monolayer. The magnitude of the shear stress and the Reynolds number, an indicator of the turbulent character of the flow, are computed as shown in "Materials and Methods" according to Sdougos et al.[41] The device allows the application of both steady and pulsatile laminar shear stress and turbulent shear stress.[40]

Shear Stress and Cyclical Stretch Apparatus

Confluent BAE and BSM cells, last fed with complete growth medium or serum-free DME 24 to 48 hours before the onset of flow, were exposed to different magnitudes and regimens of fluid shear stress by use of a previously described cone-plate viscometer specifically designed to accept standard tissue culture plates,[40] shown schematically in Fig 1.[40,41] The cells inside the viscometer were kept at a temperature of 37°C and a humidified atmosphere of 5% CO_2. Specific combinations of cone angle and rotational velocities were chosen to achieve the different flow regimens. Shear stress magnitude (τ_{ss}) and Reynolds number (R) were computed by using previously derived relations by Sdougos et al[41]:

$$R=\frac{r^2\omega\alpha^2}{12\nu}$$

$$|\tau_{ss}|\approx\left(\frac{\mu\omega}{\alpha}\right)\left[1+2.58\left(\frac{R^{3/2}}{3.5+R}\right)-0.86\left(\frac{R^{5/2}}{(3.5+R)^2}\right)\right]$$

where μ is fluid viscosity, ν is kinematic viscosity, ω is rotational velocity, and α is the cone angle. Kinematic viscosity of the medium was measured using a calibrated Canon-Fenske type viscometer (Fisher Scientific). Average Reynolds number and shear stress magnitude to which the cell population was exposed were obtained by integrating their respective values over the area of the tissue culture plate. In the steady laminar mode, a cone of 1° angle spinning at 1.4 revolutions per second was used to achieve an average shear stress magnitude of 4.1 dynes/cm^2 (low magnitude), and a cone of 1° angle spinning at 4 revolutions per second was used to achieve an average shear stress magnitude of 15 dynes/cm^2 (moderate magnitude) and Reynolds number of 0.69; in other experiments, a cone of 0.5° angle spinning at either 2.7 or 6 revolutions per second was chosen to obtain, respectively, shear stress of 15.2 dynes/cm^2 (moderate magnitude) with a Reynolds number of 0.12 and shear stress of 36 dynes/cm^2

Downloaded from http://ahajournals.org by on November 15, 2026

(elevated magnitude) with a Reynolds number of 0.26. Turbulent shear stress magnitude of 15 dynes/cm^2 was achieved by using a cone of angle 5° rotating at 4 revolutions per second and a Reynolds number of 17.2 or a cone of angle 4° rotating at 4 revolutions per second with shear stress magnitude of 15 dynes/cm^2 and a Reynolds number of 11. To achieve pulsatile shear, a modified cone varying sinusoidally in angle between 0.4° and 0.6° and rotating at 2.5 revolutions per second provided average shear stress magnitude ranging between 12 and 18 dynes/cm^2 (mean, 15 dynes/cm^2) at a frequency of 2.5 Hz (corresponding to 150 beats per minute), as previously described,[40] with corresponding Reynolds numbers of 0.07 and 0.16, respectively. Measurements of lactic dehydrogenase release showed no significant increase in the media of cells exposed to any mode of shear stress used in the present study as compared with control cells (data not shown), suggesting the absence of shear-induced cell injury.

RNA Isolation, Northern Analysis, Hybridization, and Densitometry

To isolate total RNA from BAE and BSM cells, the acid guanidium thiocyanate phenol chloroform method[43] was used. After determination of RNA purity and concentration by a spectrophotometer, 25 μg of total RNA was fractionated by use of 1.2% agarose gels containing 6% formaldehyde, 0.02 mol/L MOPS, 0.005 mol/L sodium acetate, and 0.001 mol/L EDTA. RNA was then transferred overnight by capillary action in 10× standard saline citrate (SSC, 1.5 mol/L sodium chloride and 0.15 mol/L sodium citrate [pH 7]) onto GeneScreen membranes (New England Nuclear) and immobilized by UV irradiation. Prehybridization and hybridization of the membranes were accomplished in 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 mol/L Tris-HCl (pH 7.5), 1 mol/L NaCl, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100 μg/mL denatured salmon sperm DNA, and 1 μg/mL poly(A) and poly(C). Northern blot hybridization was performed with a random-primed ^{32}P-labeled 0.7-kb *Eco*RI fragment of bovine TM cDNA,[44] 1.77-kb *Eco*RI of human TPA (ATCC No. 67585), and a 1.3-kb *Pst* I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. After hybridization, the blots were washed in 2× SSC and 1% SDS for 15 minutes at room temperature and then in 0.2× SSC and 1% SDS for 15 minutes at room temperature, followed by three washes of 20 minutes in 0.2× SSC and 1% SDS (at 63°C for TM and GAPDH and at 56°C for TPA), and exposed to x-ray film (X-Omat-AR film, Kodak) at −80°C. Autoradiograms exposed in the linear range of the x-ray films were scanned two-dimensionally by a densitometer, and the signal strength of TM or TPA mRNA was normalized for each sample with respect to the density of the corresponding GAPDH mRNA signal to compensate for any variation in RNA loading and transfer. GAPDH mRNA was chosen because its level is not affected by fluid shear stress (Reference 40 and "Results").

Western Blotting With Anti-TM Antibody

BAE monolayers were washed twice with ice-cold phosphate-buffered saline, then scraped in standard Laemmli lysis buffer containing 1.25% β-mercaptoethanol, and boiled for 5 minutes in Laemmli SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. Equal amounts of protein (100 μg) were loaded per lane and fractionated by SDS-PAGE in 10% acrylamide gels, followed by transfer using the Transblot system (Bio-Rad) at 15 V/100 mA for 12 hours at 4°C onto nitrocellulose sheets (Schleicher & Schuell) in Towbin transfer buffer (25 mmol/L Tris-HCl and 192 mmol/L glycine [pH 8.3]) containing 20% methanol. After transfer, the membrane was washed twice in 10 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl (TBS), blocked with 5% bovine serum albumin (Sigma fraction V) for 1 hour, and rinsed twice (10 minutes) in TBS and then twice in TBS with 0.5% Tween 20 (TBST). The membrane was then incubated for 2 hours with affinity-purified rabbit polyclonal anti-bovine TM antibody[44] in TBST and then washed four times (10 minutes) in TBST. Bound TM antibody was visualized using ^{125}I–protein A (30 mCi/mg, ICN) and autoradiography. Signal strength was compared by two-dimensional scanning and densitometry of TM band signal in x-rays exposed in the linear range of the autoradiography film.

Statistics

Data were expressed as mean±SEM, except where indicated. Statistical analysis was performed by ANOVA and unpaired Student's *t* test.

Results

Long-term Fluid Shear Stress Induces Cell Shape Change and Alignment in Endothelial but Not in Smooth Muscle Cells

Endothelial cell shape is known to be affected by hemodynamic forces in a specific manner, including both shear stress[40,45-47] and uniaxial strain.[48] In response to the former, the endothelium aligns in the same direction as the flow; in response to the latter, it becomes spindle shaped and assumes a perpendicular direction to the strain axis.[48] We exposed confluent monolayers of both BAE and BSM cells to steady laminar fluid shear stress of 15 dynes/cm^2 for 24 hours. Fig 2 shows that although BAE cells changed shape and alignment as previously reported,[40,45-47] BSM cell shape and direction were not affected by the shear stress stimulus, a finding consistent with a previous report.[47] This finding suggests that endothelium appears, at least at the morphological level, to be more responsive to shear forces than smooth muscle cells.

Fluid Shear Stress Decreases Expression of TM mRNA Specifically

Application of steady laminar fluid shear stress in the physiological range of either 15 (moderate magnitude) or 36 (elevated magnitude) dynes/cm^2 resulted in a time-dependent decrease in the content of TM mRNA in confluent BAE cells as seen in Northern blot analysis of total RNA (Fig 3, top). The decrease in TM mRNA was specific, since steady-state levels of GAPDH mRNA remained unaffected by shear. Densitometry was performed on a number of experiments (n=3 to 17) on the TM mRNA band normalized with respect to the corresponding GAPDH signal (Fig 3, middle and bottom). The analysis revealed that shear induced a decrease in TM mRNA from a basal control static level of unity to 0.34±0.03 (n=8, $P<.0005$) at 6 hours and to 0.36±0.29 (n=3, $P<.001$) at 9 hours for 15 dynes/cm^2 (Fig 3, middle). Under 36 dynes/cm^2, there was a trend for a mild transient increase in TM mRNA at early times between 0.5 and 2 hours, reaching up to 1.43-fold (n=3, P=NS) at 0.5 hours followed by a decrease to 0.36±0.17 (n=3, $P<.05$) at 6 hours and to 0.16±0.03 (n=3, $P<.0005$) at 9 hours (Fig 3, bottom). TM mRNA remained decreased by a similar extent even after 24 hours of continuous flow, indicating that the decrease is sustained and is not a transient phenomenon (data not shown). In contrast, exposure of BAE monolayers to low shear stress of 4 dynes/cm^2 resulted in no significant TM mRNA change when compared with static controls at times up to 12 hours (Fig 3, bottom). Exposure of BAE monolayers to this low shear stress also resulted in

Downloaded from http://ahajournals.org by on November 15, 2026

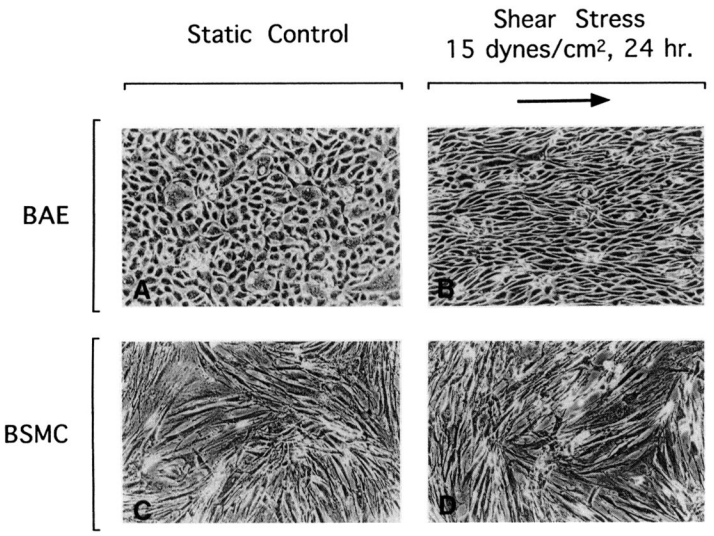


FIG 2. Photomicrographs showing that bovine aortic endothelial (BAE) cells but not bovine smooth muscle cells (BSMC) respond morphologically to flow. Confluent monolayers of BAE cells and BSMC were exposed to steady laminar shear stress of moderate magnitude (15 dynes/cm^2) for 24 hours (flow direction from left to right). As compared with static control cells, BAE cells, polygonal under static conditions in vitro, become spindle-shaped and align in the direction of flow, whereas BSMC change neither shape nor direction in response to the shear stress.

no significant morphological changes or alignment after 24 hours of flow (data not shown), a finding that is consistent with previous work.[45-47]

To determine if the shear-induced regulation of TM is a characteristic feature of endothelial cells, BSM cells that had been previously reported to express TM mRNA[49] were subjected to the same steady laminar shear of 15 dynes/cm^2. Shear stress of that magnitude and regime failed to elicit any significant changes in smooth muscle cell TM mRNA (Figure 3, middle, open squares), suggesting that the phenomenon is cell specific. The lack of TM response to shear in BSM cells is in accordance with the absence of cell shape change and alignment of these same cells shown in Fig 2.

Shear-Induced Downregulation of TM mRNA Is a Completely Reversible Process With No Evidence of Hysteresis

To assess whether the decrease in TM mRNA in response to shear is reversible on return to static no-flow conditions, BAE cells were first subjected to 6 hours of steady laminar shear at 15 dynes/cm^2, after which point flow was halted and total cellular RNA was isolated at 0.5, 3, and 6 hours. Northern analysis shows a gradual recovery of TM mRNA (Fig 4, left) to basal levels. Densitometric analysis (Fig 4, middle) of TM mRNA normalized with respect to GAPDH mRNA revealed that after 6 hours of shear stress, TM mRNA levels decreased to 30% (n=3, $P<.01$), and by 6 hours after cessation of flow, the levels had recovered to 98% of the static control levels (n=4, P=NS). Subjecting BAE cells to the same experiment but at a magnitude of 36 dynes/cm^2 yielded the same result (n=2), with recovery of TM mRNA back to its level in static control cells (data not shown). Thus, the effect of shear stress on TM mRNA appears to be completely reversible within a time frame of 6 hours.

To determine whether the endothelial cells can still respond to shear stress by decreasing TM mRNA after recovery from a period of flow, BAE monolayers were exposed to moderate shear stress (15 dynes/cm^2) for 6 hours (on), allowed to recover for 6 hours under static (no-flow) conditions (on-off), then exposed again to 6 hours of moderate shear stress (15 dynes/cm^2) (on-off-on), and harvested for Northern analysis. Fig 4, right, shows that when compared with static control cells (1.0 ± 0.05, n=7) and cells exposed to shear and then allowed to recover (on-off) (0.98 ± 0.05, n=4), cells exposed to this on-off-on protocol demonstrated decreased TM mRNA (0.24 ± 0.8, n=5) that was indistinguishable from that of BAE cells exposed to 6 hours of moderate shear stress (0.29 ± 0.07, n=5). This finding demonstrates that the TM response to shear in BAE cells is not only fully reversible but is also free of hysteresis (ie, free of memory effect).

Shear Stress Decreases Levels of Expressed TM Protein

We then explored whether the decrease in TM mRNA was also mirrored by decreases in the expression of TM protein. Western blot analysis of BAE cells exposed to 36 hours of steady laminar shear stress of 15 dynes/cm^2 using affinity-purified polyclonal antibody against bovine TM[44] revealed a significant decrease. Fig 5, left, shows the specific band at $\approx$100 kD. Densitometric analysis revealed that BAE cells exposed to shear stress expressed only 0.33 ± 0.07 of the amount of TM (n=3, $P<.005$) from BAE cells left under stationary conditions (Fig 5, right). This finding indicates that the decrease seen in TM mRNA by shear stress is also observed at the protein level.

TM mRNA Is Regulated Similarly by Steady, Pulsatile, and Turbulent Shear Stress

Fluid shear forces are inherently time varying and more complex in the living organism than those resulting from fully developed laminar flow.[39] To this end, we exposed confluent monolayers of BAE cells to pulsatile shear stress at a frequency of 2.5 Hz, varying between 12 and 18 dynes/cm^2, corresponding to a heart rate of 150 beats per minute and a variation of $\pm$20%, and to turbulent shear stress with a Reynolds number of 17.2, corresponding to a mean Kolmogorov scale of 233 μm (maximum value at the outer edge of the tissue culture surface is 350 μm).[40] The results of Northern analysis, representative of two to five separate experiments, are shown in Fig 6 and indicate no detectable difference in the extent of decreased TM mRNA expression between steady laminar, pulsatile laminar, and turbulent shear stress stimuli after 6 hours for the dynamic parameters

Downloaded from http://ahajournals.org by on November 15, 2026

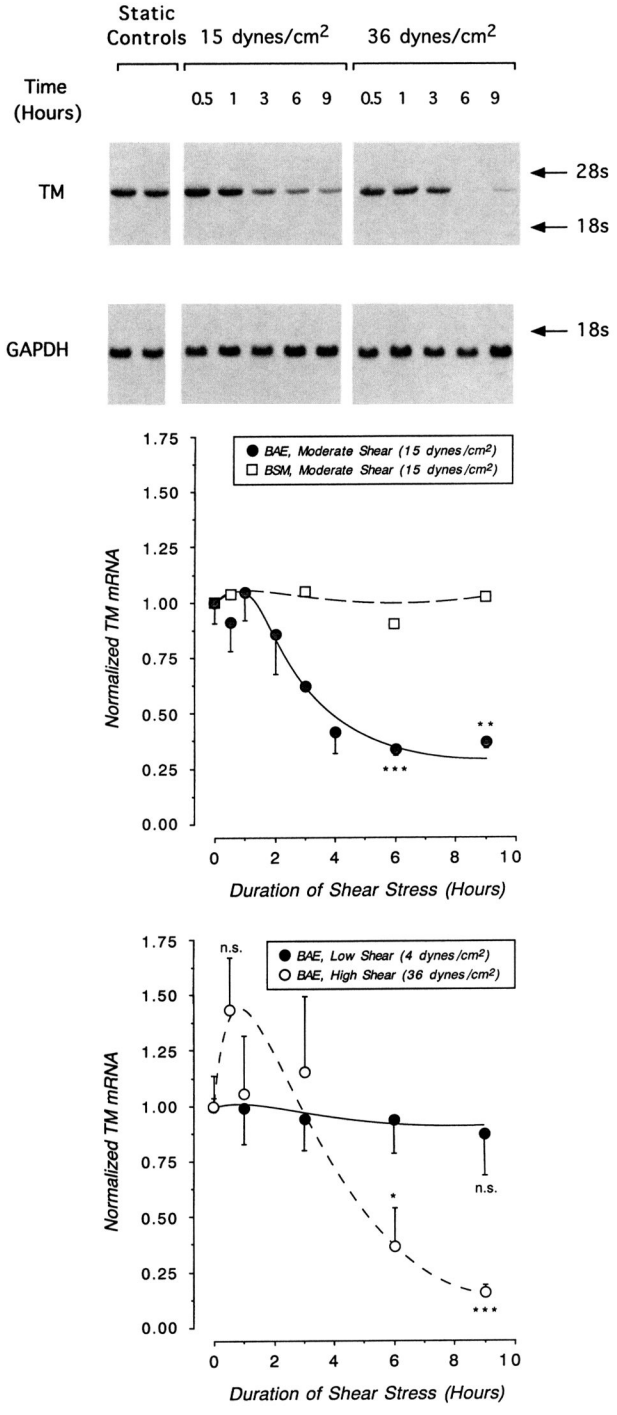


FIG 3. Steady laminar fluid shear stress in the physiological range at magnitudes of both 15 (moderate) and 36 (elevated) but not 4 (low) dynes/cm^2 induces downregulation of thrombomodulin (TM) mRNA at 6 and 9 hours. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA are not affected by fluid shear stress. Top, Northern blot shows total RNA isolated from bovine aortic endothelial (BAE) cells showing TM and GAPDH mRNA content with respect to duration of exposure to steady laminar shear stress of moderate (15 dynes/cm^2) and elevated (36 dynes/cm^2) magnitude for increasing duration. Middle, Graph shows densitometric analysis of TM mRNA content normalized with respect to GAPDH for BAE (●) and BSM (□) cells at moderate shear stress of 15 dynes/cm^2. Note the downregulation of TM mRNA in response to shear in BAE but not BSM cells. Bottom, Graph shows densitometric analysis of TM mRNA content normalized with respect to GAPDH for BAE cells at low shear stress of 4 dynes/cm^2 (●) and elevated shear stress of 36 dynes/cm^2 (○). Note the absence of response at low shear stress and the accentuated TM downregulation at elevated shear as compared with moderate shear for >6 hours (*P<.05, **P<.001, and ***P<.0005).

chosen here. All three shear stimuli chosen were characterized by having the same absolute mean shear stress magnitude of 15 dynes/cm^2. Similar TM downregulation was also observed with a cone of angle 4° and a mean Kolmogorov scale of 186 μm.

Differential Regulation of TM and TPA mRNA by Fluid Shear Stress

Since TPA mRNA has previously been reported to be increased by fluid shear stress in HUVE cells when exposed to 25 dynes/cm^2,[30] it was of interest to determine the relation between the TM and TPA changes in mRNA expression induced by fluid shear stress. It was also important to determine whether BAE behaved in the same fashion as HUVE cells in response to shear stimulus. Accordingly, mRNA collected from BAE cells exposed to either 15 or 36 dynes/cm^2 was analyzed by Northern blot with a TPA cDNA probe. Densitometry of the normalized signal indicates that at 15 dynes/cm^2, where TM is downregulated to 30% (Fig 3, middle), TPA is increased by approximately threefold compared with static control levels (Fig 7). However, TPA mRNA levels increase very dramatically (≈22-fold) at the higher shear magnitude of 36 dynes/cm^2 (Fig 7), whereas TM mRNA remains downregulated at 36 dynes/cm^2, even further (at 16%) than at 15 dynes/cm^2. The finding of dramatically higher response of TPA to shear of 36 compared with 15 dynes/cm^2 is qualitatively similar to the previously reported increase in HUVE TPA mRNA at 25 but not 4 dynes/cm^2, reported by Diamond and colleagues[29,30] and similar to our recent observations with basic fibroblast growth factor.[50]

Discussion

TM is a surface receptor for thrombin found mostly on endothelial cells but also has been recently shown to be expressed in smooth muscle cells[49] and polymorphonuclear leukocytes.[51] Among richly vascularized tissues, TM mRNA is found at its highest concentration in the lung and probably in greater quantities in continuous than in fenestrated endothelium.[52] Because of its potent action in catalyzing the activation of protein C, TM is thought to play an important role in regulating the antithrombotic activity of the endothelial cell by multiple mechanisms, including activation of protein C, inactivation of factors Va and VIIIa, and internalization of thrombin.[9] Recent work[14] also implicates TM in antagonizing the activation of endothelial cells by thrombin. Since the endothelium is constantly exposed to flow and the resulting fluid shear stress in vivo, it is important to determine the role of fluid dynamic forces on the expression of TM. We used an in vitro cone-plate viscometer[40] to apply physiological fluid shear stress under 4, 15, and 36 dynes/cm^2 and have shown a clear and significant dose-dependent decrease in TM mRNA, which is mirrored by a decrease in TM protein under moderate shear, in BAE cells. In contrast, BSM cells exposed to 15 dynes/cm^2 did not alter their level of TM

Downloaded from http://ahajournals.org by on November 15, 2026

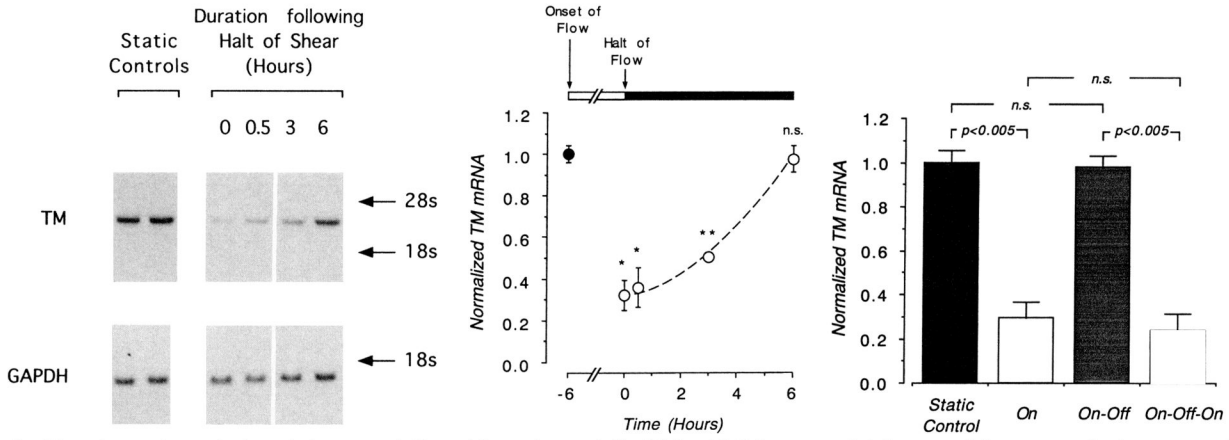


FIG 4. The shear stress–induced downregulation of thrombomodulin (TM) mRNA is a completely reversible process that recovers after the cessation of flow and is free of hysteresis. Left, bovine aortic endothelial (BAE) cells were subjected to 6 hours of steady laminar shear stress of 15 dynes/cm^2, followed by cessation of flow and total RNA analysis at 0, 0.5, 3, and 6 hours. Middle, Graph shows densitometric analysis of three or four separate experiments demonstrating near complete recovery of TM mRNA by 6 hours after cessation of the shear stimulus to their static control levels (*P<.01 and **P<.001). Right, Bar graph shows separate BAE cell monolayers exposed to 6 hours of steady laminar shear stress of moderate magnitude (15 dynes/cm^2) (on), followed by 6 hours of static no-flow conditions (on-off), and further followed by another 6 hours of steady laminar shear stress of moderate magnitude (15 dynes/cm^2) (on-off-on). Densitometric analysis of TM mRNA normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reveals complete recovery in on-off experiments (n=4) to static control levels (n=7, P=NS) and similar TM downregulation between on (n=5) and on-off-on (n=5, P=NS). This demonstrates the absence of irreversible injury to BAE cells and also lack of hysteresis in the TM response to shear.

Downloaded from http://ahajournals.org by on November 15, 2026

expression in any significant manner, indicating that the observed phenomenon is specific to endothelial cells. It is worthwhile to mention that shear stress might initially result in a transient increase in TM mRNA expression, which is, however, later significantly decreased, a phenomenon previously seen in other shear-sensitive genes such as platelet-derived growth factor (PDGF)-B and endothelin-1 (ET-1).[40,50] It is likely that the downregulation of TM mRNA is more important than the transient peak, since long-term (36-hour) exposure of BAE cells to 15 dynes/cm^2 resulted in decreased TM protein. However, it is important to note that the measured changes in TM mRNA and protein expression reported here may not entirely reflect changes in TM activity that could be observed in vivo.

Areas of low flow, low wall shear stress (≈4 dynes/cm^2), or flow recirculation are characterized by a prolonged particle residence time, which has been proposed to increase the likelihood of interaction between activated blood-borne elements and the vessel wall[20,39]

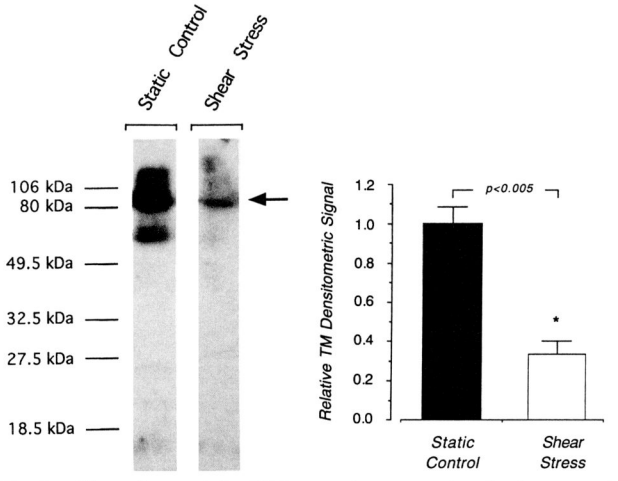


FIG 5. Thrombomodulin (TM) protein expression is decreased by long-term moderate steady laminar shear stress of 15 dynes/cm^2. Left, Confluent bovine aortic endothelial monolayers were exposed to steady laminar shear stress of 15 dynes/cm^2 for 36 hours and then lysed and processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis using rabbit affinity-purified polyclonal anti-bovine TM antibody.[44] Note that TM migrates at ≈100 kD under reducing conditions. Right, Bar graph shows densitometric analysis of three separate experiments exposed to moderate shear stress for 36 hours, demonstrating a threefold decrease in TM protein (P<.005), consistent with the observed decreased TM mRNA.

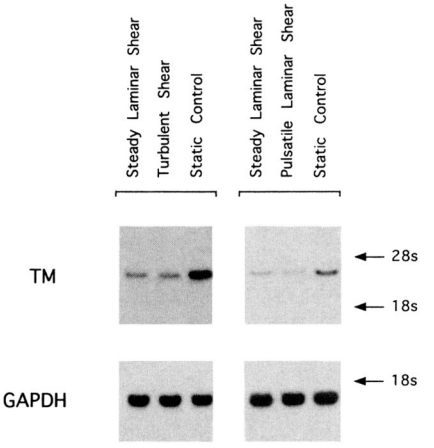


FIG 6. Effect of varying the dynamic character of the shear stress stimulus on thrombomodulin (TM) mRNA downregulation. Confluent bovine aortic endothelial monolayers were exposed to shear stress with a mean magnitude of 15 dynes/cm^2 for 6 hours in three separate regimens: (1) steady laminar shear, (2) turbulent shear, and (3) pulsatile shear (varying sinusoidally between 12 and 18 dynes/cm^2 at 2.5 Hz). Note that the right upper blot is underexposed compared with the other blots but that all three regimens showed a similar decrease in TM mRNA. These results are representative of two to five separate experiments. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase.

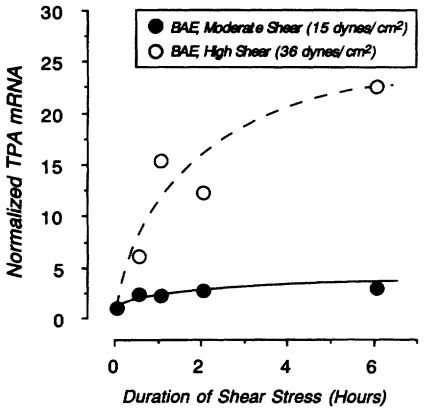


FIG 7. Graph showing the response of tissue-type plasminogen activator (TPA) mRNA to steady laminar fluid shear stress in bovine aortic endothelial (BAE) monolayers exposed to moderate (15 dynes/cm^2) (●) and elevated (36 dynes/cm^2) (○) magnitude. Note the mild increase with respect to time at 15 dynes/cm^2 and the more dramatic increase at 36 dynes/cm^2, reaching more than 20-fold at 9 hours. This result illustrates the differential regulation of TPA and TM mRNA depending on shear stress magnitude and suggests, with Fig 3, a switch in endothelial phenotype from a predominantly antithrombotic state (high TM, low TPA) at low and moderate shear to a fibrinolytic one (low TM, high TPA) at high shear.

and thereby possibly increase the local risk of thrombosis. On the other extreme, shear stress of pathologically elevated magnitude also appears to directly induce von Willebrand factor/GPIIb-IIIa–mediated platelet aggregation and is associated with an increased risk of thrombus formation.[53,54] Thus, the multiple effects of hemodynamic forces on endothelial and platelet function suggest that fluid shear stress may be a critical factor in the regulation of thrombosis.

The findings reported here demonstrate that cells constantly exposed to flow and shear stress eventually express lower steady-state levels of TM mRNA and protein than do cells under conditions of low or no flow. Such a property suggests that TM may serve a protective role in cells under static conditions, which may be prone to a higher probability of thrombosis and clot formation.[20,39] The decrease in TM mRNA by shear stress was demonstrated to be entirely reversible within 6 hours after the cessation of shear stress and to also be hysteresis free, suggesting that the cells have not undergone an irreversible injurious insult and further pointing to the possibility that such a mechanism would be dynamically responsive in vivo to changing local hemodynamic conditions.

The response of TM to fluid shear stress did not appear to discriminate between laminar steady, laminar pulsatile, and turbulent regimens of equal mean magnitude of 15 dynes/cm^2. This finding is similar to that previously described for ET-1 and PDGF-B chain[40,50] and suggests that TM mRNA is more sensitive to the low-frequency component (mean magnitude) of the shear stimulus than to the higher frequency but smaller components seen in the turbulent or pulsatile regimens used here. The limitation of this result must also be recognized, since the dynamic characteristics of the chosen shear stimuli were arbitrary and may not represent conditions observed in vivo. It is possible that other time-varying shear conditions of higher frequency or magnitude may elicit a different result, particularly in light of other experiments demonstrating a different endothelial behavior under turbulent[55] or pulsatile[56] shear. Our finding may be interpreted as further evidence that the BAE cell may be acting as a viscoelastic structure[57,58] and as such may be less sensitive to small higher frequency components of the shear stimulus.

The downregulation of TM mRNA and protein with application of fluid shear stress was observed to be concomitant with an increase in TPA mRNA, especially at the elevated magnitude of 36 dynes/cm^2. This result demonstrates that the shear responsiveness of the TPA gene originally described in HUVE cells[30] is also present in BAE cells. Together, the TM and TPA patterns of regulation suggest a switch in the phenotype of the endothelial cell from a predominantly antithrombotic and partially antifibrinolytic role[59,60] characterized by high TM and low TPA expression under static conditions to a fibrinolytic phenotype with decreased TM and increased TPA expression under conditions of flow, especially at the elevated level of shear stress. This is further strengthened by reports documenting that TM accelerates the thrombin-mediated inactivation of single-chain uPA and can thereby play an antifibrinolytic role in addition to its well-known antithrombotic role.[59,60]

The decrease in TM mRNA reached a steady-state level as soon as 6 hours after the onset of shear stress and also recovered within the same time period back to its previous level. Since the half-life of TM mRNA has been previously estimated at ≈8.9 hours,[61] it appears unlikely for the shear-induced downregulation to be solely mediated by decreases in TM transcription without alterations in message stability such as has been described for ET-1.[62]

Although the mechanism of TM regulation by shear is not at the present understood, it is possible to rule out cAMP involvement, since fluid shear stress of 20 dynes/cm^2 in BAE cells did not affect cAMP levels by any significant measure.[62] Furthermore, cAMP analogues and cAMP-elevating agents have been implicated in increased TM expression,[34] whereas shear stress induces the opposite effect. Protein kinase C is probably not involved, because in our system we were not able to detect significant protein kinase C activation as determined by translocation of enzyme activity or immunoreactivity of the α and β isozyme[62] at 20 dynes/cm^2, a magnitude at which TM mRNA is downregulated. Although shear is known to induce EDRF release[31,32] and, by an autocrine action of the latter, increase cGMP, Hirokawa and Aoki[63] reported no changes in TM activity with db-cGMP administration. Finally, chelation of intracellular calcium by 10 μmol/L quin 2-AM, to prevent shear-induced transient increases in $[Ca^{2+}]_i$,[23-25] did not prevent the shear-induced downregulation of TM mRNA (authors' unpublished observation). The final pathway from the transduction of the shear stress on the endothelial surface to the altered expression of TM remains to be elucidated.

In summary, our data show that TM, an important negative regulator of thrombosis with partial antifibrinolytic properties, is reversibly regulated by mechanical forces in an endothelial cell-specific, magnitude-dependent, and hysteresis-free manner and in an inverse relation with respect to TPA. These results add to the role of mechanical forces and fluid shear stress in the

Downloaded from http://ahajournals.org by on November 15, 2026

regulation of the complex array of interactions that regulate thrombosis and hemostasis at the surface of the endothelial cell.

Acknowledgments

This study was supported by a Whitaker Foundation Grant (Dr Izumo) and by a Johnson & Johnson Foundation Research Grant (through the Harvard-MIT Health Sciences and Technology Division) (Dr Malek). Dr Malek is supported by the National Institutes of Health Medical Scientist Training Program. Dr Izumo is an Established Investigator of the American Heart Association. We thank G. Soff for providing BSM cells.

References

1. Esmon CT. The regulation of natural anticoagulant pathways. *Science.* 1987;235:1348-1352.
2. Prescott M, McIntyre TM, Zimmerman GA. The role of platelet-activating factor in endothelial cells. *Thromb Haemost.* 1990;64: 99-103.
3. Radomski RA, Palmer RMJ, Moncada S. Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br J Pharmacol.* 1987;92:181-187.
4. Busse R, Luckhoff A, Bassenge E. Endothelium-derived relaxant factor inhibits platelet activation. *Arch Pharmacol.* 1987;336: 566-571.
5. Nguyen BL, Saitoh M, Ware AJ. Interaction of nitric oxide and cGMP with signal transduction in activated platelets. *Am J Physiol.* 1991;261:H1043-H1052.
6. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev.* 1993;73:161-195.
7. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. *J Clin Invest.* 1991;88:1067-1072.
8. Sprengers ED, Kluft C. Plasminogen activator inhibitors. *Blood.* 1987;69:381-387.
9. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J Biol Chem.* 1989;264:4743-4746.
10. Comp PC. Hereditary disorders predisposing to thrombosis. In: *Progress in Hemostasis and Thrombosis.* Philadelphia, Pa: Grune & Stratton Inc; 1986:71.
11. Gomi K, Zushi M, Honda G, Kawahara S, Matsuzaki O, Kanabayashi T, Yamamoto S, Maruyama I, Suzuki K. Antithrombotic effect of recombinant human thrombomodulin on thrombin-induced thromboembolism in mice. *Blood.* 1990;75:1396-1399.
12. Taylor FB, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. Protein C prevents the coagulopathic and lethal effects of *Escherichia coli* infusion in the baboon. *J Clin Invest.* 1987;79: 918-925.
13. Conway EM, Boffa MC, Nowkowski B, Steiner-Mosonyi M. An ultrastructural study of thrombomodulin endocytosis: internalization occurs in clathrin-coated and non-coated pits. *J Cell Physiol.* 1992;151:604-612.
14. Parkinson JF, Bang NU, Garcia JGN. Recombinant human thrombomodulin attenuates human endothelial cell activation by human thrombin. *Arterioscler Thromb.* 1993;13:1119-1123.
15. Kamiya A, Bukhari R, Togawa T. Adaptive regulation of wall shear stress optimizing vascular tree function. *Bull Math Biol.* 1984;46: 127-137.
16. LaBarbera M. Principles of design of fluid transport systems in zoology. *Science.* 1990;249:992-1000.
17. Sherman TF, Popel AS. The cost of departure from optimal radii in microvascular networks. *J Theor Biol.* 1989;136:245-265.
18. Kamiya A, Togawa T. Adaptive regulation of wall shear stress to flow change in the canine carotid artery. *Am J Physiol.* 1980;239:H14-H21.
19. Langille L, O'Donnell F. Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. *Science.* 1986;231:405-407.
20. Asakura T, Karino T. Flow patterns and spatial distribution of atherosclerotic lesions in human coronary arteries. *Circ Res.* 1990; 66:1045-1066.
21. Davies PF, Tripathi SC. Mechanical stress mechanisms and the cell: an endothelial paradigm. *Circ Res.* 1993;72:239-245.
22. Olesen S, Clapham D, Davies PF. Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. *Nature.* 1988; 331:168-170.
23. Shen J, Luscinskas FW, Connolly A, Dewey F, Gimbrone M. Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. *Am J Physiol.* 1992;262:C384-C390.
24. Mo M, Eskin S, Schilling WP. Flow-induced changes in Ca^{++} signalling in vascular endothelial cells: effect of shear stress and ATP. *Am J Physiol.* 1991;260:H1698-H1707.
25. Dull RO, Davies PF. Flow modulation of agonist (ATP)-response (Ca^{2+}) coupling in vascular endothelial cells. *Am J Physiol.* 1991; 261:H149-H154.
26. Ziegelstein RC, Cheng L, Capogrossi MC. Flow-dependent cytosolic acidification of vascular endothelial cells. *Science.* 1992; 268:656-659.
27. Frangos JA, Eskin SG, McIntire LV, Ives CL. Flow effects on prostacyclin production by cultured human endothelial cells. *Science.* 1984;227:1477-1479.
28. Bagyalakshmi A, Frangos J. Mechanism of shear-induced prostacyclin production in endothelial cells. *Biochem Biophys Res Commun.* 1989;158:31-37.
29. Diamond S, Eskin S, McIntire L. Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. *Science.* 1989;243:1483-1485.
30. Diamond SL, Sharefkin JB, Dieffenbach C, Frasier-Scott K, McIntire LV, Eskin SG. Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. *J Cell Physiol.* 1990;143:364-371.
31. Cooke JP, Rossitch E, Andon NA, Loscalzo J, Dzau CJ. Flow activates an endothelial channel to release an endogenous nitrovasodilator. *J Clin Invest.* 1991;88:1663-1671.
32. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. *FASEB J.* 1989;3:2007-2018.
33. Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, Murphy TJ. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J Clin Invest.* 1992;90:2092-2096.
34. Wiler-Guettler H, Yu K, Soff G, Gudas LJ, Rosenberg RD. Thrombomodulin gene regulation by cAMP and retinoic acid in F9 embryonal carcinoma cells. *Proc Natl Acad Sci U S A.* 1992;89: 2155-2159.
35. Dittman WA, Kimdad T, Majerus PW. Transcription of thrombomodulin mRNA in mouse hemagioma cells is increased by cycloheximide and thrombin. *Proc Natl Acad Sci U S A.* 1989;86: 7179-7182.
36. Bartha K, Brisson C, Archipoff G, de la Salle C, Lanza F, Cazenave J-P, Beretz A. Thrombin regulates tissue factor and thrombomodulin mRNA levels and activities in human saphenous vein endothelial cells by distinct mechanisms. *J Biol Chem.* 1993; 268:421-429.
37. Moore KL, Esmon CT, Esmon NL. Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture. *Blood.* 1989; 73:159-165.
38. Lentz SR, Sadler JE. Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest.* 1991;88:1906-1914.
39. Goldsmith HL, Turitto VT. Rheological aspects of thrombosis and haemostasis: basic principles and applications. *Thromb Haemost.* 1986;55:415-435.
40. Malek A, Izumo S. Physiological fluid shear stress causes down-regulation of endothelin-1 mRNA in bovine aortic endothelium. *Am J Physiol.* 1992;263:C389-C396.
41. Sdougos HP, Bussolari SR, Dewey CF. Secondary flow and turbulance in a cone-plate device. *J Fluid Mech.* 1984;138:379-404.
42. Reilly C, Fritze LM, Rosenberg RD. Heparin-like molecules regulate the number of epidermal growth factor receptors on vascular smooth muscle cells. *J Cell Physiol.* 1988;136:23-32.
43. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 1987;162:156-159.
44. Jackman RW, Beeler DL, VanDeWater L, Rosenberg RD. Characterization of a thrombomodulin cDNA reveals structural similarity to the low density lipoprotein receptor. *Proc Natl Acad Sci U S A.* 1986;83:8834-8838.
45. Remuzzi A, Dewey ZF, Davies P, Gimbrone MA. Orientation of endothelial cells in shear fields in vitro. *Biorheology.* 1984;21: 617-630.
46. Levesque MJ, Nerem RM. The elongation and orientation of cultured endothelial cells in response to shear stress. *J Biomech Eng.* 1985;107:341-347.
47. Eskin SG, McIntire LV. Hemodynamic effects on atherosclerosis and thrombosis. *Semin Thromb Hemost.* 1988;14:170-174.
48. Shirinsky VP, Antonov AS, Birukov KG, Sobolevsky AV, Romanov YA, Kabaeva NV, Antonova GN, Smirnov VN. Mechano-chemical

Downloaded from http://ahajournals.org by on November 15, 2026

control of human endothelial orientation and size. *J Cell Biol.* 1989; 109:331-339.
49. Soff GA, Jackman RW, Rosenberg RD. Expression of thrombomodulin by smooth muscle cells in culture: different effects of tumor necrosis factor and cyclic adenosine monophosphate on thrombomodulin expression by endothelial cells and smooth muscle cells in culture. *Blood.* 1991;77:515-518.
50. Malek AM, Gibbons GH, Dzau VJ, Izumo S. Fluid shear stress differentially modulates expression of genes encoding basic fibroblast growth factor and platelet-derived growth factor B-chain in vascular endothelium. *J Clin Invest.* 1993;92:2013-2021.
51. Conway EM, Nowakowski B, Steiner-Mosonyi M. Human neutrophils synthesize thrombomodulin that does not promote thrombin-dependent protein C activation. *Blood.* 1992;80:1254-1263.
52. Ford VA, Stringer C, Kennel SJ. Thrombomodulin is preferentially expressed in Balb/c lung microvessels. *J Biol Chem.* 1992;267: 5446-5450.
53. Kroll MH, Hellums JD, Guo Z, Durante W, Razdan K, Hrbolich JK, Schafer AI. Protein kinase C is activated in platelets subjected to pathological shear stress. *J Biol Chem.* 1993;268:3520-3524.
54. Alevriadou BR, Moake JL, Turner N, Ruggeri ZM, Folie BJ, Phillips MD, Schreiber AB, Hrinda ME, McIntire LV. Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. *Blood.* 1993;81:1263-1276.
55. Davies PF, Remuzzi A, Gordon E, Dewey F Jr, Gimbrone MA Jr. Turbulent fluid shear stress induces vascular endothelial cell turnover. *Proc Natl Acad Sci U S A.* 1986;83:2114-2117.
56. Hsieh HJ, Li NQ, Frangos JA. Pulsatile and steady flow induces c-fos expression in human endothelial cells. *J Cell Physiol.* 1993; 154:143-151.
57. Herman IM, Brant AM, Warty VS, Bonaccorso J, Klein EC, Kormos RL, Borovetz HS. Hemodynamics and the vascular endothelial cytoskeleton. *J Cell Biol.* 1987;105:291-302.
58. Adams DS. Mechanisms of cell shape change: the cytomechanics of cellular response to chemical environment and mechanical loading. *J Cell Biol.* 1992;117:83-93.
59. Majerus PW, DeMunk GAW, Groeneveld E, Rijken DC. Acceleration of the thrombin inactivation of single chain urokinase-type plasminogen activator (pro-urokinase) by thrombomodulin. *J Clin Invest.* 1991;88:1680-1684.
60. Molinari A, Gorgietti C, Lansen J, Orsini G, Faioni EM, Mannucci PM. Thrombomodulin is a cofactor for thrombin degradation of recombinant single-chain urokinase plasminogen activator "in vitro" and in a perfused rabbit heart model. *Thromb Haemost.* 1992;67:226-232.
61. Dittman WA, Kumada T, Sadler EJ, Majerus PW. The structure and function of mouse thrombomodulin. *J Biol Chem.* 1988;263: 15815-15822.
62. Malek AM, Greene AL, Izumo S. Regulation of endothelin-1 gene by fluid shear stress is transcriptionally mediated and independent of protein kinase C and cAMP. *Proc Natl Acad Sci U S A.* 1993; 90:5999-6003.
63. Hirokawa K, Aoki N. Regulatory mechanisms for thrombomodulin in human umbilical vein endothelial cells in vitro. *J Cell Physiol.* 1991;147:157-165.

Downloaded from http://ahajournals.org by on November 15, 2026