Fluid Shear Stress on Endothelial Cells Modulates Mechanical Tension across VE-Cadherin and PECAM-1

Daniel E. Conway,1 Mark T. Breckenridge,2 Elizabeth Hinde,3 Enrico Gratton,2 Christopher S. Chen,2 and Martin A. Schwartz1,4,∗

1Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville VA, 22908, USA
2Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104, USA
3Laboratory for Fluorescence Dynamics, Biomedical Engineering Department, University of California, Irvine, Irvine, CA 92697, USA
4Departments of Medicine (Cardiology) and Cell Biology, Yale University, New Haven, CT 06511, USA

Summary

Fluid shear stress (FSS) from blood flow acting on the endothelium critically regulates vascular morphogenesis, blood pressure, and atherosclerosis [1]. FSS applied to endothelial cells (ECs) triggers signaling events including opening of ion channels, activation of signaling pathways, and changes in gene expression. Elucidating how ECs sense flow is important for understanding both normal vascular function and disease. EC responses to FSS are mediated in part by ion channels, activation of signaling pathways, and changes in gene expression. To further examine its behavior, we measured its dynamics by fluorescent recovery after photobleaching (FRAP) (Figure 1C). Recovery curves for VECadTS were identical to wild-type VE-cadherin, indicating normal dynamics. Additionally, we measured intermolecular FRET by cotransfected cells with two VECadTS constructs, one containing mutant nonfluorescent teal and the other mutant nonfluorescent venus. FRET was much less than for VECadTS and did not differ from the analogous tailless constructs (Figure S1C), indicating that intermolecular FRET is low and, together with the results below, is independent of tension.

Confluent monolayers of bovine aortic endothelial cells (BAECs) transfected with VECadTS were untreated or incubated with inhibitors of myosin activation, either 10 µM ROCK inhibitor Y-27632 or 10 µM myosin light chain kinase inhibitor ML7. Cells expressing either the tailless control or the soluble module were also examined. In untreated cells in serum, VECadTS exhibited the expected zipper-like junctional morphology, whereas the tailless construct exhibited a more linear morphology (Figure 1D). Cells treated with the myosin inhibitors also had linear junctions (Figure 1D and data not shown). FRET index images of junctional VECadTS in untreated cells showed lower FRET compared to junctional tailless sensor and the cytoplasmic soluble module (Figure 1E), indicating that VECadTS in junctions is under tension. Similar results were obtained when the VE-cadherin tension sensor was expressed in VE-cadherin−/− cells, suggesting that the presence of endogenous cadherin in the BAEC does not affect the tension on the VE-cadherin sensor (data not shown). Treatment with Y27632 and/or ML7 increased FRET, indicating a decrease in tension (Figure 1E). Similar results were obtained for fixed cells, indicating that fixation of the sensor, under high or low tension, does not affect FRET (Figures S3E and S3F). As an additional control, FRET was measured fluorimetrically for detergent-solubilized constructs. The FRET efficiency of detergent-solubilized VECadTS and tailless constructs, which are presumably under no tension, were both ~30% (Figure S1D), as reported for the soluble module [6]. Thus, FRET for VECadTS in solution is identical to the tailless construct. Together, these results show that for cells in normal growth medium without flow, VE-cadherin is under myosin-dependent tension.

Results

Development of a VE-Cadherin Tension Sensor

We initially screened expression and localization of constructs in which the tension sensor module was inserted into multiple sites within VE-cadherin (data not shown). The optimal construct had the tension sensor between the p120-binding domain and the β-catenin-binding domain in the cytoplasmic tail (Figure 1A). We also constructed a zero-force (high FRET) control in which the C-terminal β-catenin-binding domain was deleted. The VE-cadherin tension sensor (VECadTS), expressed in VE-cadherin−/− endothelial cells, localized to cell junctions and distributed similarly to endogenous VE-cadherin in human umbilical vein endothelial cells (HUVECs) (Figure 1B). To test its function in flow sensing, VE-cadherin−/− cells were reconstituted with VECadTS or wild-type VE-cadherin with a C-terminal Venus fluorescent protein and exposed to 15 dynes/cm² shear stress for 24 hr. VECadTS restored alignment similarly to wild-type VE-cadherin, whereas the tailless control was inactive (Figure S1A, available online, quantified in Figure S1B). The VE-cadherin tension sensor is therefore functional in flow sensing.

To further examine its behavior, we measured its dynamics by fluorescent recovery after photobleaching (FRAP) (Figure 1C). Recovery curves for VECadTS were identical to wild-type VE-cadherin, indicating normal dynamics. Additionally, we measured intermolecular FRET by cotransfected cells with two VECadTS constructs, one containing mutant nonfluorescent teal and the other mutant nonfluorescent venus. FRET was much less than for VECadTS and did not differ from the analogous tailless constructs (Figure S1C), indicating that intermolecular FRET is low and, together with the results below, is independent of tension.

Confluent monolayers of bovine aortic endothelial cells (BAECs) transfected with VECadTS were untreated or incubated with inhibitors of myosin activation, either 10 µM ROCK inhibitor Y-27632 or 10 µM myosin light chain kinase inhibitor ML7. Cells expressing either the tailless control or the soluble module were also examined. In untreated cells in serum, VECadTS exhibited the expected zipper-like junctional morphology, whereas the tailless construct exhibited a more linear morphology (Figure 1D). Cells treated with the myosin inhibitors also had linear junctions (Figure 1D and data not shown). FRET index images of junctional VECadTS in untreated cells showed lower FRET compared to junctional tailless sensor and the cytoplasmic soluble module (Figure 1E), indicating that VECadTS in junctions is under tension. Similar results were obtained when the VE-cadherin tension sensor was expressed in VE-cadherin−/− cells, suggesting that the presence of endogenous cadherin in the BAEC does not affect the tension on the VE-cadherin sensor (data not shown). Treatment with Y27632 and/or ML7 increased FRET, indicating a decrease in tension (Figure 1E). Similar results were obtained for fixed cells, indicating that fixation of the sensor, under high or low tension, does not affect FRET (Figures S3E and S3F). As an additional control, FRET was measured fluorimetrically for detergent-solubilized constructs. The FRET efficiency of detergent-solubilized VECadTS and tailless constructs, which are presumably under no tension, were both ~30% (Figure S1D), as reported for the soluble module [6]. Thus, FRET for VECadTS in solution is identical to the tailless construct. Together, these results show that for cells in normal growth medium without flow, VE-cadherin is under myosin-dependent tension.

*Correspondence: martin.schwartz@yale.edu
Effects of Flow

Next, monolayers of BAECs expressing VECadTS were exposed to fluid shear stress and FRET measured. Surprisingly, we observed a rapid increase in FRET, indicating a decrease in force, that was evident at 30 s, the earliest time point we could measure, and persisted for at least 30 min (Figure 1F). To understand this result, we first tested whether β-catenin might dissociate from VE-cadherin in response to flow. Immunoprecipitation of VE-cadherin did not reveal any decrease in associated β-catenin (Figure S2A), arguing against this possibility. However, to examine this idea more rigorously, we constructed a mutant VECadTS in which the β-catenin-binding domain was truncated and replaced with the C-terminal actin-binding region of α-catenin (VEcad-αcat-TS; Figure S2B). Under resting conditions, VECad-αcat-TS had higher FRET, indicating less tension compared to wild-type VE-cadherin (Figure S2C). This result is consistent with the more linear junctions formed by this construct (data not shown and [8]) and may be related to the deletion of the vinculin-binding site in α-catenin [9]. However, application of flow still
triggered a further increase in FRET (Figure S2C). Thus, dissociation of VE-cadherin from β-catenin does not mediate the reduction in tension. VE-cadherin−/− ECs reconstituted with the VE-cadherin β-catenin fusion (lacking the tension sensor insert) also supported realignment (Figure S2D), indicating that the β-catenin linkage is not required for mechanotransduction beyond connecting VE-cadherin to actin.

We then measured total intracellular junctional tension in monolayers of cells on arrays of elastic posts, whose deflection can be used to calculate both cell-substrate traction forces and intracellular junction forces [10]. Cells on posts showed similar flow activation of src family kinases (data not shown) and alignment in the direction of flow [11], indicating that this substratum does not interfere with flow responses. Onset of shear stress triggered a rapid decrease in both traction forces and cell-cell forces by around 25% (Figures 1G and 1H). Note that cells on coverslips did not show loss of actin stress fibers or focal adhesions (data not shown). We conclude that flow triggers decreases in total cell-cell force, which very likely mediate the decreased tension on VE-cadherin.

**Fluorescence Lifetime Measurements**

To confirm these results from intensity measurements, we also measured the fluorescence lifetime of the donor fluorophore as an alternative method for determining FRET efficiency, based on the fact that FRET reduces the lifetime of the donor. This technique readily determines true FRET efficiency, based on the fact that FRET reduces the lifetime of the donor fluorophore as an alternative method for determining FRET efficiency. Coexpression showed minimal intermolecular FRET, which was unchanged between full-length and tailless sensors (Figure S2C). Additionally, we observed no significant difference in FRET between PECAM-1 and the tailless control, indicating that tension across PECAM is negligible under static conditions.

**Effects of Flow on PECAM-1 Tension**

Next, confluent monolayers of cells expressing PECAM-TS (Figure 2D) were subjected to 15 dynes/cm² shear stress for various times. We observed a rapid decrease in FRET that was evident at 30 s and was sustained for at least 30 min, indicating an increase in tension. No changes were observed for PECAM-TS increased significantly at junctional regions, indicating that there is a single population that shifts in FRET between static and shear stress conditions, indicating that the changes under shear stress equally affect all junctional VE-cadherin molecules rather than a subpopulation (Figure S2F). Comparison of these values to the previously determined calibration [6] showed that average tension on VE-cadherin decreased from 2.4 to 1.8 nN/molecule after flow.

**Development of a PECAM-1 Tension Sensor**

To develop a PECAM-1 tension sensor, we again made multiple constructs in which the tension sensor module was inserted at different sites and then screened for expression and localization (data not shown). The optimal construct (PECAM-TS; Figure 2A) contained the sensor module in the cytoplasmic tail immediately before exon 15, which was reported to mediate cytoskeletal anchoring [12]. PECAM-TS localized to cell-cell junctions, where it distributed similarly to endogenous PECAM-1 in HUVEC (Figure 2B). Expression in PECAM-1−/− cells restored cells’ ability to align in flow, whereas the tailless control was inactive (Figure S3A, quantification in Figure S3B). FRAP measurements indicated that dynamics for PECAM-TS were similar to PECAM-1 with a C-terminal Venus (Figure 2C). We also made versions with individually mutated fluorescent proteins to measure intramolecular FRET. Coexpression showed minimal intermolecular FRET, which was unchanged between full-length and tailless sensors (Figure S2C). Additionally, we observed no significant difference in FRET between PECAM-TS and the tailless control, indicating that tension across PECAM is negligible under static conditions.
the tailless control (data not shown). To confirm these results and obtain true FRET efficiency, we also measured donor lifetime. FRET efficiency in unstimulated cells was identical to the tailless control and flow for 2 min resulted in decreased FRET at cell-cell contacts but no change in nonjunctional areas (Figure 2E). Thus, tension on PECAM-1 increased on the same timescale as VE-cadherin tension decreased. Comparison to the published calibration [6] revealed that average tension/junctional PECAM-1 molecule after flow was 2.0 pN/molecule. Similar to VE-cadherin, histograms of PECAM-TS FRET efficiency indicated that the change between static and shear stress conditions is due to a shift of a single population (Figure S3D). To test whether these changes in PECAM-1 and VE-cadherin are related, we measured VE-cadherin tension in PECAM-1−/− cells and PECAM-1 tension in VE-cadherin−/− cells. Changes in tension for each sensor required expression of the other receptor (Figures 3A and 3B), indicating that effects of flow on tension across these receptors are interdependent.

Lack of Directionality
We analyzed images to determine whether changes in tension for VE-cadherin and PECAM-1 showed any polarity with respect to the direction of flow. Junctions that were perpendicular or parallel to the direction of flow were identified and the FRET index calculated (Figures 3C and 3D). After 2 min of flow, no difference between perpendicular and parallel junctions was observed.

PECAM Cytoskeletal Association
We next considered whether the increased tension across PECAM-1 might be due to altered associations with cytoskeletal components. To detect specific interactions, we expressed PECAM-3xFLAG in BAECs, treated the cells with peptides from the 54 kDa intermediate filament protein vimentin (Table S1). To confirm this result, we probed PECAM-3xFLAG eluates for vimentin. Shear stress increased the vimentin in Flag immunoprecipitations from transfected cells, whereas untransfected cells showed only low and nonreproducible background (Figure 4A). Immunoprecipitation of endogenous PECAM from HUVECs, in which peptide elution was not feasible, frequently yielded high vimentin backgrounds in all samples, probably as a result of the insolubility of vimentin. Nevertheless, in the experiments with lower backgrounds, increased vimentin in sheared cells was observed (data not shown). We therefore turned to functional assays to further validate this interaction.

We previously observed that endothelial cells plated on anti-PECAM-1 antibodies adhere and spread (D.E.C. and M.A.S., unpublished data); this system was therefore used to test a possible vimentin-PECAM-1 connection. BAECs expressing human PECAM-1 were seeded onto glass coverslips coated with PECAM-1 antibody clone 1.3, which binds to the extracellular domain of human PECAM-1 and mimics ligation [13]. Transfected cells adhered, whereas little adhesion was observed with untransfected cells or transfected cells on nonimmune IgG (Figure S4B). Importantly, depletion of vimentin with two different siRNA sequences (Figure S4C) reduced adhesion (Figure 4B). Vimentin knockdown did not affect cell adhesion and spreading on fibronectin (Figure S4B), demonstrating specificity. Cos-7 cells, which do not express PECAM-1, were transfected with wild-type PECAM, PECAM-TS, or PECAM-TL and plated on PECAM-1 antibody-coated coverslips (Figure 4C). Whereas PECAM-TS supported adhesion similarly to WT PECAM, PECAM-TL, which lacks exons 15 and 16 of the cytoplasmic tail, supported cell attachment only weakly. Thus, the C-terminal sequences in the cytoplasmic domain are required for both application of force

![Figure 3. Interdependence of Force Changes on PECAM and VE-Cadherin Expression and Junctional Orientation](image-url)
Requirement for Vimentin

We next tested whether vimentin is required for the flow-dependent increase in tension on PECAM. BAECs expressing PECAM-TS were treated with siRNA to deplete vimentin and then exposed to shear stress (Figure 4D). Vimentin knockdown did not affect expression of endogenous PECAM-1 or VE-cadherin (Figure 4D) or the localization of PECAM-1 or VE-cadherin to junctions (data not shown). However, vimentin knockdown completely prevented the increase in force on PECAM. To investigate the requirement for myosin-dependent tension, control BAECs (without vimentin knockdown) were pretreated with both Y-27632 and ML7. Blockade of myosin activation also prevented the increased force on PECAM after flow (Figure 4E). Interestingly, vimentin knockdown had no effect on the tension across VE-cadherin before or after flow (Figure 4F). Cells with vimentin knockdown also failed to align in the direction of flow (Figure 4G).

Discussion

We report here the development and validation of biosensors to measure tension across VE-cadherin and PECAM-1. We found that in control endothelial cells in standard growth medium, VE-cadherin was under substantial myosin-dependent tension, while tension on PECAM-1 was undetectable. Onset of shear stress triggered a rapid decrease in tension on VE-cadherin by about 25%, concomitant with a general relaxation of cell-cell and cell-matrix forces of the same magnitude. By contrast, tension on PECAM-1 increased. Flow also triggered an association between PECAM and vimentin, which was required for tension on PECAM-1. Changes across VE-cadherin and PECAM were mutually interdependent, such that flow-dependent changes in each required expression of the other. However, vimentin knockdown blocked the
force increase on PECAM-1 without affecting tension across VE-cadherin, indicating that the changes in forces are not strictly coupled. This result suggests instead that PECAM-1 has both tension-dependent and -independent roles in shear stress signaling.

The involvement of a PECAM-vimentin link in flow sensing fits well with several published results. The PECAM-1 cytoplasmic domain was reported to associate with the vimentin- linker proteins γ-catenin (plakoglobin) and desmoplakin [12]. Imaging GFP-vimentin revealed flow-induced strains in the vimentin network, which were frequently maximal at cell-cell junctions [14]. Vimentin knockout mice showed impaired short-term flow-mediated dilation [15, 16] and longer-term flow-dependent vessel remodeling [17], similar to PECAM-1 knockout mice [5, 18]. Our results are therefore consistent with a wide range of in vitro and in vivo data. However, the increased association of PECAM-1 with vimentin raises a number of questions. Does PECAM-1 trigger local vimentin polymerization versus new association with preformed filaments? Are there linkers that connect PECAM-1 to vimentin filaments? How do vimentin filaments connect to the actin cytoskeleton to transmit force? These questions will be addressed in future work.

We also observed decreased tension on VE-cadherin after flow. This change was not due to release of VE-cadherin from the cytoskeleton but instead to a general flow-induced decrease in cell-cell tension (Figures 1G and 1H). A recent report using traction force microscopy found no changes in intracellular forces in the plane parallel to the surface after 30 min of flow [19]. However, this technique is inherently different than measuring intercellular junctional forces with micropost array detectors (mPADs); in the traction force measurements, the intracellular forces are separated out into parallel and perpendicular forces, and the parallel forces may derive from sources other than cell-cell junctions.

These experiments used cells that were fixed prior to FRET analysis, based on control experiments showing no significant differences in FRET in live versus fixed cells (Figures S3E and S3F). The analysis also involved averaging the entire junctional signal from multiple cells. This analysis facilitated detection of statistically significant effects in populations but did not allow for analysis of temporal and spatial dynamics. These issues will be addressed in future work.

In agreement with our results showing no differences in tension across VE-cadherin or PECAM based on the orientation of the junction to flow (Figures 3C and 3D), the prior study of intercellular forces also found no differences in directionality at 30 min [19]. Analysis of the earliest signaling events downstream of the junctional complex showed no evidence for polarity [2, 20]. This lack of polarity or directionality provides further evidence against the idea that changes in force across VE-cadherin and PECAM-1 are due to direct transfer of force from the apical domain to cell-cell junctions through the cytoskeleton.

A similarly designed tension sensor for E-cadherin expressed in epithelial cells also showed high tension under normal culture conditions [21]. However, they reported that cell surface E-cadherin outside of cell-cell junctions was under substantial tension. Using FLIM, which yields true FRET efficiency and is less subject to artifact than intensity measurements, we detected no tension on VE-cadherin expressed outside of cell junctions (Figure 11, Figure S2E). The reasons for these differences are currently unknown.

A previous model of flow sensing proposed that forces are transmitted through the cytoskeleton to the cell-cell junctions [7]. The identification of PECAM-1 as a mechanosensor for flow fit well with this view [2], and the current results provide a critical confirmation for the concept that shear stress results in increased force on PECAM-1 to initiate downstream signaling [2]. However, several features of these new data are difficult to reconcile with passive force transfer through the cytoskeleton to points of cell adhesion. Instead, the data indicate that flow triggers association of PECAM with vimentin, which transmits myosin-generated forces to PECAM. This model implies the existence of an upstream mechanosensor that initiates these cytoskeletal rearrangements. In this regard, it may be relevant that traction force measurements by Hur et al. showed that the cell-cell tension and the intracellular tension in endothelial cells under laminar shear flow is almost one order of magnitude larger than the value required to passively balance the shear stresses [19]. Thus, weak forces from flow trigger application of the much stronger myosin-dependent forces to PECAM-1. This type of amplification mechanism is common in biochemical signaling pathways but, to our knowledge, has not been observed in mechanotransduction. Elucidation of the molecular mechanism upstream of the changes in tension on PECAM-1 and VE-cadherin will be an important goal for future work.

Experimental Procedures

Complete experimental procedures are described in the Supplemental Experimental Procedures.

Reagents and Cells

Bovine endothelial cells (Coriell Institute), VE-cadherin−/− and PECAM-1−/− endothelial cell lines [2], and HUVECs (gift of Brett Blackman, University of Virginia) were used in the indicated experiments. Cells were subjected to 15 dynes/cm² shear stress using a parallel plate chamber [2].

DNA Constructs

The previously described tension sensor module (TSMod) [6] was inserted into the cytoplasmic domains of mouse VE-cadherin and human PECAM-1 cDNA to generate the respective tension sensors. Tailless constructs were generated by using a TSMod with a stop codon immediately after the venus sequence.

Knockdown of vimentin was performed by inserting the knockdown sequence into the psuer plasmid (Oligoengine) according to manufacturer instructions. The vimentin target sequences used were AGGCCAAGCAG GAGTCAA (sequence 1) and AGGAATGGTACAAGTCCAA (sequence 2).

FRET

FRET analysis was performed with nonlinear spectral bleed-through corrections as previously described [6].

Measurement of Traction Force and Junctional Tension

mPADs were fabricated as described [22]. Tugging and traction forces were calculated as described [10]. After bisection of the monolayer along cell-cell junctions, the intercellular tugging force is calculated to balance the net traction force per section [10, 23].

FLIM Acquisition

Cells were exposed to shear stress, fixed, and analyzed for fluorescence lifetime. The FLIM data were acquired and processed by the SimFCS software developed at the Laboratory for Fluorescence Dynamics (http://www.lfd.uci.edu). In the manuscript, we use a region of interest analysis to determine the FRET efficiency of the PECAM and VE cadherin tension sensor under static and shear conditions. For each sensor, the published TSMod FRET efficiency versus force calibration curve was adjusted as described [6].

Statistics

Experiments with three or more conditions were analyzed with one-way ANOVA, which was performed using Newman-Keuls multiple comparison
test. Experiments with two samples were analyzed using Student’s t test. The threshold for significance was taken as p < 0.05. Data are represented as mean ± SEM.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.04.049.

Acknowledgments

This work was funded by NIH grant RO1 HL75092 to M.A.S.; RO1 HL73305 and the RESBIO Technology Resource for Polymeric Biomaterials to C.S.C.; USPHS training grant 5T32-HL07284 and AHA Postdoctoral Fellowship to D.E.C.; and NIH-P41-RR03155, P41 GM103540 and NIH P50-GM076516 to E.G. We thank Brenton Hoffman (Duke University) and Carsten Grashoff (Max Planck Institute of Biochemistry, Germany) for helpful discussions; Tyler Ross (Yale University) for analysis of PECAM attachment images; and Lukas Tamm (University of Virginia), Alpha Yap (University of Queensland, Australia), and Peter Newman (Blood Center of Wisconsin) for generously providing reagents.

Received: October 28, 2012
Revised: March 14, 2013
Accepted: April 18, 2013
Published: May 16, 2013

References