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Mapping the Dynamics of Shear Stress—Induced Structural Changes in Endothelial Cells

Rosalind E. Mott and Brian P. Helmke

Department of Biomedical Engineering and Robert M. Berne Cardiovascular Research Center
University of Virginia, Charlottesville, Virginia, USA

Abstract

Hemodynamic shear stress regulates endothelial cell biochemical processes that govern cytoskeletal contractility, focal adhesion dynamics, and extracellular matrix assembly. Since shear stress causes rapid strain focusing at discrete locations in the cytoskeleton, we hypothesized that shear stress coordinately alters structural dynamics in the cytoskeleton, focal adhesion sites, and extracellular matrix on a time scale of minutes. Using multi-wavelength 4-D fluorescence microscopy, we measured the displacement of rhodamine-fibronectin and of GFP-labeled actin, vimentin, paxillin, and/or vinculin in aortic endothelial cells before and after onset of steady unidirectional shear stress. In the cytoskeleton, the onset of shear stress increased actin polymerization into lamellipodia, altered the angle of lateral displacement of actin stress fibers and vimentin filaments, and decreased centripetal remodeling of actin stress fibers in both subconfluent and confluent cell layers. Shear stress induced the formation of new focal complexes and reduced the centripetal remodeling of focal adhesions in regions of new actin polymerization. The structural dynamics of focal adhesions and the fibronectin matrix varied with cell density. In subconfluent cell layers, shear stress onset decreased the displacement of focal adhesions and fibronectin fibrils. In confluent monolayers, the direction of fibronectin and focal adhesion displacement shifted significantly towards the downstream direction within one minute after onset of shear stress. These spatially coordinated rapid changes in the structural dynamics of cytoskeleton, focal adhesions, and extracellular matrix are consistent with focusing of mechanical stress and/or strain near major sites of shear stress-mediated mechanotransduction.

Keywords

mechanotransduction; cytoskeleton; extracellular matrix; focal adhesion

INTRODUCTION

The endothelium regulates a number of physiological vascular functions such as maintenance of vessel tone, prevention of thrombosis, promotion of fibrinolysis, and initiation of angiogenesis. The spatial and temporal pattern of shear stress generated by local hemodynamics is a critical factor in determining endothelial function (7,11). In particular, regions of laminar shear stress are less susceptible to atherosclerosis development than are regions with disturbed hemodynamic profiles.

Although the initial events that trigger cellular adaptation to the local hemodynamic profile remain unclear, focal adhesion sites have been implicated as intracellular locations where

mechanosignaling is initiated (3,27,29). A decentralization hypothesis proposes that force transmitted from the cell surface through the cytoskeleton modulates biochemical activity associated with mechanotransduction (13), but direct measurement of these local forces has not yet been possible. If hemodynamic forces are transmitted through cytoskeleton to adhesion sites, then the structural dynamics associated with cytoskeleton—focal adhesion interactions and extracellular matrix remodeling will serve as indicators of changes in mechanical interactions.

Both in vivo and in vitro, endothelial cell (EC) shape and cytoskeletal structure align parallel to the direction of shear stress in regions of unidirectional flow that are less susceptible to atherogenesis (8,10,21). In live cell microscopy measurements, onset of shear stress induces heterogeneous patterns of mechanical “strain focusing” within the intermediate filament network near the basal surface of ECs that could cause changes in conformation or organization of molecules within focal adhesion sites (15). Indeed, unidirectional steady shear stress in vitro induces dynamic alignment of focal adhesions in EC monolayers (6) and polarized assembly in migrating ECs (20).

These quantitative measurements of dynamic structural rearrangements suggest locations of mechanical stimuli of biochemical activity at the subcellular length scale. For example, both mean traction force against the substrate and Rho GTPase activity are increased in migrating, subconfluent ECs during the first 30 min after onset of shear stress (28). The shear stress—induced regulation of Rho activity depends on cell density and the degree of cell-cell contact. In confluent monolayers, the time course of Rho activity is biphasic with an initial decrease and a subsequent increase (33). In contrast, subconfluent human umbilical vein ECs exhibit a peak in RhoA activation at 5 min after onset of shear stress and decreased activity at 15 min (34). In addition to regulating Rho activity, shear stress stimulates a transient increase in Rac activity within minutes after application to confluent EC monolayers (32). Rac activity peaks 30 min after onset of shear stress, and the distribution of activated Rac becomes polarized preferentially to the downstream end of the cell. Rac is a key regulator of actin polymerization and focal complex formation, and Rho is a central regulator of acto-myosin contractility, stress fiber formation, and focal adhesion maturation. Therefore, the shear stress—induced dynamic regulation of these signaling molecules may serve to alter intracellular mechanical strain and force transmission throughout the cytoskeleton. However, whether intracellular deformation and mechanical interactions are differentially regulated in migrating single cells and confluent monolayers in response to shear stress remains unsolved.

The physical connection from the cytoskeleton to the extracellular matrix through focal adhesion sites may also contribute to shear stress—induced extracellular matrix remodeling. Fibronectin expression by endothelial cells is transiently suppressed immediately following the onset of shear stress but is significantly increased after 48 h, at which time bundled fibrils exist that are aligned parallel to the flow direction (31). In vivo, fatty streaks in the artery wall exhibit upregulated levels of fibronectin in the subendothelial basement membrane, indicating that fibronectin is related to pathological mechanisms in atheroprone regions of disturbed or low magnitude hemodynamic shear stress (25). Control of fibronectin assembly and remodeling in these regions is likely to be a complex system involving the contractile state of the cell, the regulation of de novo fibronectin synthesis, and the shear stress—mediated regulation of matrix metalloprotease expression. Although the endothelium in vivo usually appears intact in histological sections of atherosclerotic lesions, increased permeability at these locations suggests the existence of dysfunctional intercellular junctions. As a result, it is not clear whether intracellular force distribution relevant to mechanosensing mechanisms is altered.

Although endothelial cells in vivo exhibit different structure and gene expression at atherosclerosis-prone locations, current hypotheses for intracellular mechanotransmission do not account for differences between regions of disrupted cell structure and areas of intact atherosclerosis-resistant monolayers. The goal of this study was to compare quantitatively in subconfluent and confluent endothelial cell layers the dynamic structural response of the cytoskeleton, focal adhesions, and extracellular matrix to onset of unidirectional laminar shear stress. We hypothesized that onset of shear stress results in focused force transmission that alters within minutes the relative structural dynamics of the actin cytoskeleton, the intermediate filament network, the focal adhesions sites, and the fibronectin matrix, and these structural dynamics in response to shear stress depend on the state of cell-cell contact. Finally, we show for the first time simultaneous dynamic changes that suggest intracellular and extracellular strain focusing near focal adhesion sites involved in triggering mechanotransduction signaling networks.

MATERIALS AND METHODS

Cell Culture, Transfection, and Fluorescent Labeling

Bovine aortic ECs at passages 10-15 were cultured in complete growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 2 mM L-glutamine, 50 u/ml penicillin G, and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were plated onto No. 1-1/2 coverslips marked with 0.1-µm diameter fluorescent microspheres (Invitrogen) as described previously (14). Transient transfections implemented Lipofectin (Invitrogen) in order to express pEGFP-actin (Clontech, Mountain View, CA) and paxillin-DsRed2 (a gift from A. F. Horwitz, Univ. of Virginia), mRFP-β-actin (pCMV-mRFP-actin, a gift from E. Fuchs, Rockefeller Univ.) and EGFP-vimentin (pEGFP-hVIM-Myc, a gift from R. D. Goldman, Northwestern Univ.), or EGFP-vinculin (pEGFP/V1-1066, a gift from S. W. Craig, Johns Hopkins Univ.) (4). Fibronectin (Sigma-Aldrich, St. Louis, MO) was labeled using the EZ-Label™ Rhodamine Protein Labeling Kit (Pierce Biotechnology, Rockford, IL). In order to image fluorescently labeled fibronectin, glass coverslips were coated with rhodamine-fibronectin (Rd-FN) at 20 µg/ml for 30-60 min and were rinsed with PBS for 5-30 min. Cells were plated and allowed to grow and assemble fibronectin fibrils for 16 or 48 h. In all experiments, cells were exposed to 15 dyn/cm² steady laminar shear stress in a parallel plate chamber (Bioptechs, Butler, PA) at 37 °C perfused with complete growth medium. The pH was maintained at 7.4 by equilibration with 5% CO₂—95% air at 100% relative humidity. For studies with latrunculin A, ECs were grown to confluence on glass coverslips coated with Rd-FN and then treated with complete growth medium containing 100 nM latrunculin A (Calbiochem, La Jolla, CA) for 1-6 h. Treated cells were either subjected to 15 dyn/cm² shear stress with medium containing 100 nM latrunculin A or were fixed with 4% paraformaldehyde. Fixed cells were stained with FITC-phalloidin (Sigma), vinculin monoclonal antibody (clone hVIN-1, Sigma), or VE-cadherin polyclonal antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA). Affinity purified, Cy3 conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (Westgrove, PA).

Image Acquisition

4-D images were obtained with a DeltaVision restoration microscopy system (Applied Precision, Issaquah, WA) consisting of an Olympus IX70 microscope with mercury lamp illumination, a 60X/1.4 NA objective, emission wavelengths of 528 nm and 617 nm, and a cooled CCD camera (Micromax, Princeton Instruments, Trenton, NJ). Image stacks of 10 optical slices spaced 200 nm apart were obtained in 2.5-min intervals for consecutive 15 min periods before and after onset of shear stress.

Fields of view for image acquisition were chosen in central regions of the parallel plate flow chamber where fully developed laminar flow existed. Each field of view included at least one randomly selected EC that was transiently over-expressing fluorescent fusion proteins at moderate levels. In DIC microscopy images, cell morphology and behavior was identical in transfected and non-transfected ECs, and transfected ECs that were incorporated into confluent monolayers were indistinguishable from adjacent non-transfected ECs. In separate studies, immunofluorescent labeling of vinculin and paxillin confirmed that focal adhesions in fixed transfected ECs were similar in shape, size, and number to those in non-transfected ECs (data not shown).

Image Processing

Images were deconvolved using an experimentally measured point spread function and a constrained iterative algorithm (*softWorx*, Applied Precision) and were exported in TIFF format. Microsphere positions were used to subtract coverslip movement from the time-lapse data. All analysis was performed for intervals before and after onset of shear stress. Stress fiber termini and focal adhesion sites were tracked manually in *ImageJ* (NIH) (1) or by using *IDL* (ITT Visual Information Systems, Boulder, CO) algorithms adapted from Crocker and Grier (5) and *Matlab* (MathWorks, Natick, MA) algorithms. In order to measure the degree of spatial displacement in time-lapse images of the fibronectin matrix, a displacement index (DI) was computed as described previously (16). Briefly, the degree of overlap between images $I(x, y, t_i)$ and $I(x, y, t_j)$ acquired at times t_i and t_j , respectively, was computed as the product moment correlation coefficient (PMCC):

$$\text{PMCC}(t_i, t_j) = \frac{\text{Cov}[I(x, y, t_i), I(x, y, t_j)]}{(\text{Var}[I(x, y, t_i)], \text{Var}[I(x, y, t_j)])^{1/2}},$$

where $\text{Cov}(I)$ and $\text{Var}(I)$ are the spatial covariance and variance, respectively, computed from the images. The displacement index (DI) was computed as

$$\text{DI}(t_i, t_j) = 1 - \text{PMCC}(t_i, t_j).$$

Displacement index values were computed for subregions of the images of the fibronectin matrix and were represented by a color map displaying the computed values for each subregion. Overlay images from two time points were generated in *Photoshop* (Adobe Systems, San Jose, CA).

Statistical Analysis

The mean displacement magnitudes of all visible focal adhesion sites in each cell were used to compute the overall mean $\pm$ SEM, and mean displacements before and after onset of shear stress were compared (paired t test, $p < 0.05$). The mean displacement magnitudes of all DI values from image subregions were used to compute the overall mean $\pm$ SEM, and mean displacements before and after onset of shear stress were compared (paired t test, $p < 0.05$). In order to determine whether displacement of stress fiber termini was significantly correlated with nearby focal adhesion site displacement, a t test was performed with the following statistic:

$$t = \frac{r \sqrt{N - 2}}{\sqrt{1 - r^2}},$$

where r is the Pearson's correlation and N is the number of samples. The correlation was considered significantly greater than zero if the t test allowed rejecting the null hypothesis that $r=0$ with >95% confidence. Analysis of displacement directions was performed as described previously (15). Briefly, a Rayleigh test was performed to determine whether displacement direction was uniformly distributed on the circle with >95% confidence (9), and mean resultant direction $\pm$ circular variance was reported if the distribution was non-uniform.

RESULTS

Onset of shear stress induces rapid lamellipodium and focal complex formation

In subconfluent or confluent cell layers expressing EGFP- β -actin and paxillin-DsRed2, time-lapse image sequences were acquired every 2.5 min for 10-15 min intervals before and after onset of steady unidirectional shear stress. Edge ruffling and lamellipodium extension were evaluated in two-color overlay images (Fig. 1). In quiescent confluent monolayers, comparison of EGFP- β -actin images at the beginning (red) and end (green) of a 7.5-min interval indicated low levels of constitutive edge activity (Fig. 1 A). However, onset of shear stress induced bursts of new protrusions in random directions around the cell periphery within 2.5 min, and comparison of actin morphology just before (red) to 7.5 min after (green) onset of shear stress revealed multiple locations of significant lamellipodium activity (Fig. 1 B). Many of these protrusions were not stable (see **Supplementary Data**, Movie 1), but some persisted through the 10 min interval and served to increase projected cell area by $3.8 \pm 2.5\%$ ($n = 17$). In subconfluent cell layers with visible baseline levels of lamellipodial protrusions, onset of shear stress increased the number of protrusions within 2.5 min. The formation of lamellipodia gradually became predominant in the downstream edge of the cells by 15 min. At this time, focal complexes containing paxillin-DsRed2 (Fig. 2, red) were detectable within the new lamellipodia outlined by EGFP- β -actin. After 1 h exposure to shear stress, these focal complexes matured into larger focal adhesions within stable lamellae.

Shear stress induces shifts in the lateral displacement of actin stress fibers

Not only did shear stress alter the peripheral morphology by the formation of new protrusions, but it also induced shifts in the direction of the lateral displacement of pre-existing stress fibers. In order to determine whether flow-induced stress fiber displacement occurred preferentially in downstream regions of ECs, lateral stress fiber displacement was tracked in upstream and downstream regions of the cell for 15-min intervals before and during shear stress. Cells were divided into upstream and downstream regions delineated by a straight line perpendicular to the flow direction, through the center of the nucleus. Manual tracking revealed that a subpopulation of stress fibers underwent lateral displacements under no-flow conditions. The displacement path was smooth and included few direction changes. Within 2.5 min after onset of shear stress, the direction of these lateral displacements changed dramatically, as actin filaments rapidly switched displacement directions. The spatial pattern of flow-induced stress fiber displacement was not different in the upstream region of the cell than in the downstream region. Changes in the angle of displacement induced by onset of shear stress were as large as 180° and had a mean absolute value of $100^\circ \pm 77^\circ$ ($n=15$ stress fibers from 8 cells). Although the pattern of displacement was spatially heterogeneous in the cells, the shear induced changes tended to promote the lateral displacement of stress fibers in the downstream direction, since 67% of stress fibers were displaced in the downstream direction. Additionally, the onset of shear stress served to increase the displacement rate of 60% of the examined stress fibers (see **Supplementary Data**, Movies 2-3).

Lateral displacements in the vimentin network are similar to those of actin stress fibers

Previous studies showed that onset of shear stress induces localized deformation in the intermediate filament network indicated by directed displacement on the micrometer scale

(15). Since the measured lateral displacement of actin stress fibers was also of order 1 μm , we measured relative displacement between intermediate filaments and microfilaments by co-transfecting ECs with EGFP-vimentin and mRFP- β -actin (Fig. 3). Comparison of images just before (red) and 8 min after (green) onset of shear stress illustrates changes in filament position or morphology in the vimentin (Fig. 3 A, C, E) and actin (Fig. 3 B, D, F) cytoskeletons. The displacement of vimentin filaments closely paralleled that of actin stress fibers in both direction and magnitude in both subconfluent and confluent cells. Interestingly, shear stress-induced lateral displacements often correlated with the direction of the new lamellipodial protrusions (Fig. 3 C-D, arrows).

Shear stress reduces the centripetal remodeling of stress fibers and their terminal focal adhesions adjacent to areas of new actin polymerization

Since onset of shear stress induced dramatic changes in the displacement patterns of cytoskeletal networks, we hypothesized that shear stress-induced motion represented mechanical changes that were transmitted to physically connected focal adhesions. Under no-flow conditions, a sub-population of stress fibers and mature focal adhesions on the periphery often underwent a steady process of centripetal displacement toward the cell body. This phenomenon has been observed previously in the focal adhesions of fibroblasts and requires actin stress fiber contractility (30). After onset of shear stress, centripetal remodeling ceased in both stress fibers containing EGFP- β -actin (Fig. 4) and focal adhesion sites containing paxillin-DsRed2 (Fig. 5). As an illustrative example, manual tracking of three typical stress fiber termini and their associated focal adhesion sites (Fig. 6) demonstrated that the cumulative displacement of these structures reached a plateau after onset of shear stress. Furthermore, centripetal displacement of stress fiber termini was highly correlated with the displacement of their associated focal adhesions ($r = 0.97 \pm 0.03$, $n = 6$, $p < 0.005$). Interestingly, the suppression of stress fiber and focal adhesion displacement was associated with regions of shear stress-induced lamellipodium formation (Fig. 4, arrows). Within 5 μm of newly formed shear stress-induced lamellipodia, $69 \pm 6\%$ of focal adhesion sites exhibited reduced displacement after onset of shear stress ($n = 300$ focal adhesions in 3 cells). The effect of flow onset on displacement in the whole-cell bulk population of adhesion sites varied with cell density. Displacement magnitude was computed during consecutive 15-min intervals before (*static*) and after (*flow*) onset of shear stress for subconfluent cells plated for 16 hrs and confluent cells plated for 48 hrs. Computational tracking of adhesion sites containing EGFP-vinculin indicated a significant decrease in the mean displacement of adhesion sites in 92% of subconfluent cells ($n = 13$ cells, $p < 0.005$) after onset of shear stress (Fig. 7). In contrast, the mean adhesion site displacement during the no-flow interval in confluent cells ($n = 13$ cells) was not significantly different from that during the interval after onset of shear stress ($p = 0.6$). Although shear stress did not induce a statistically significant change in the mean displacement magnitude of adhesion sites, the direction of adhesion site displacement was oriented significantly in the downstream direction (mean resultant angle = $-37^\circ \pm 22^\circ$, $n = 378$ in 12 cells, $p < 0.0001$) during the first minute after onset of shear stress (Fig. 8 B). The displacement direction again became uniform over the remainder of the observed 15 minute interval (Fig. 8 C).

Shear stress induces rapid shifts in the deformation of fibronectin fibrils in the extracellular matrix that are associated with changes in adhesion dynamics and are dependent upon cell density

In order to determine whether shear stress-induced structural dynamics in the cytoskeleton and focal adhesion sites was transmitted to the extracellular matrix, cells expressing EGFP-vinculin were plated on coverslips coated with rhodamine-labeled fibronectin (Rd-FN) for 16 h to develop mature focal adhesions and a fibrillar fibronectin matrix. Subconfluent cells assembled the Rd-FN into short fibrils ($< 15 \mu\text{m}$ long) that terminated on focal adhesions containing EGFP-vinculin (Fig. 9 A). A displacement index (DI) ranging in value from 0 (blue) to 1 (red) was

computed to quantify the spatial distribution of relative displacement of these structures. Under no-flow conditions, DI values computed from images of the fibronectin matrix varied spatially, reaching maxima along the cell periphery (Fig. 9 B). In these subconfluent cell layers, onset of shear stress served to significantly decrease DI (mean change in DI = -0.12 ± 0.02 , $n = 8$ fields of view, $p=0.001$), and it remained reduced for at least 15 min (Fig. 9 C). This pattern of reduced DI values correlated with the significant decrease in the mean adhesion displacement values (Fig. 7). By manually tracking the lengths of fibronectin fibrils, we found that under no-flow conditions fibril length increased by as much as 34% in a 15-min interval. After onset of shear stress, fibril lengths stabilized, indicating that shear stress inhibited additional mechanical strain in the fibronectin matrix composed of short fibrils.

In order to measure the impact of shear stress on the fibronectin matrix underlying a confluent monolayer of cells, ECs were plated on coverslips coated with Rd-FN for 48 h, and images were acquired every 2.5 min for 15 min intervals before and after onset of shear stress (see **Supplementary Data**, Movie 4). Rd-FN was assembled into a dense network of fibrils that often extended under multiple cells and was more interconnected than the early matrix observed under subconfluent cells. Comparison of Rd-FN fibril positions at the beginning (red) and end (green) of a no-flow interval demonstrated that the fibril displacement patterns were heterogeneous (Fig. 10 A), and the displacement direction was uniformly distributed (Rayleigh test, $p=0.11$). During the first minute after onset of shear stress, the fibril displacement was directed primarily in the downstream direction (Fig. 10 B) with a mean resultant angle of $-7^\circ \pm 21^\circ$ with respect to the axis parallel to shear stress (Rayleigh test, $p<0.0001$, $n=260$ fibrils in 15 fields of view). During the remainder of the 15-minute flow interval, this downstream directionality was reduced (mean resultant angle = $-46^\circ \pm 42^\circ$) but still significant (Rayleigh test, $p<0.0001$) as the displacement pattern of the fibrils gradually became more spatially heterogeneous (Fig. 10 C). Thus, onset of shear stress induced an initial trend of downstream fibril displacement followed by a complex spatial pattern of fibril displacement under confluent monolayers of ECs. This behavior of the fibronectin matrix at the onset of shear stress correlated well with that of focal adhesions and provided evidence for force transmission from the inside to the outside of the cell (see Fig. 11 and **Supplementary Data**, Movie 5).

Latrunculin treatment deteriorates flow-induced displacement magnitude over time

To examine the role of the actin cytoskeletal network in force transmission to the fibronectin matrix through focal adhesions, the actin cytoskeleton was perturbed pharmacologically. Actin polymerization was inhibited by the addition of 100 nM latrunculin A (lat A), a toxin that blocks actin polymerization by binding actin monomer, for 1–6 h to the cell culture media. This concentration disassembled the actin cytoskeletal network, focal adhesions, and adherens junctions over time without causing the cells to round up and detach from the matrix. The effects of lat A on the actin cytoskeleton on a confluent monolayer were gradual and less visually obvious than those on subconfluent cells (unpublished observations). There existed few structural differences between untreated and treated cells after 1 h, but the effects of lat A treatment were apparent by 4 h (**Supplementary Data**, Fig. S1). At 1 h, the confluent cells had a reduced number of mature focal adhesions (Fig. S1 H), but their actin dense peripheral bundles, stress fibers and adherens junctions remained largely intact (**Fig. S1 E and K**). At 4 h, the junction structure was compromised; this effect could be seen by the retraction of VE-cadherin-labeled cell edges with the creation of space between cells (**Fig. S1 F, I, L**).

The direction and magnitude of fibronectin matrix fibrils were measured to determine whether lat A treatment impacted shear stress—induced displacement. Under static conditions the displacement directions were uniformly distributed (**Fig. S2 A**, Rayleigh Test, $p=0.23$). During the first minute after onset of shear stress, the fibril displacement was directed primarily in the downstream direction (**Fig. S2 B**) with a mean resultant angle of $-21^\circ \pm 29.8^\circ$ with respect to

the axis parallel to shear stress (Rayleigh test, $p < 0.0001$, $n = 304$ fibrils in 8 fields of view). During the remainder of the 15-min flow interval, this downstream directionality was again reduced (**Fig. S2 C**) but still significant (Rayleigh test, $p < 0.0001$) as the displacement pattern of the fibrils gradually became more spatially heterogeneous. Throughout the lat A treatment period, the characteristic shear stress-induced downstream displacement of the fibronectin matrix (**Fig. S2**) remained similar to the directional profile measured without latrunculin (**Fig. 10 E-G**). However, the magnitude of displacement decreased with increasing duration of lat A exposure. At 1 h (**Fig. 12 A**) shear stress displaced the matrix underlying an intact monolayer by distances on the order of micrometers. The monolayer structure was degraded and the displacement magnitudes decreased (**Fig. 12 B**) with increasing lat A exposure. The magnitude of displacement appeared to be dependent upon fibrillar architecture, so the significance of the change in displacement magnitude was tested by subjecting the same field of view to shear onset at 1 h post—lat A treatment and at 4 h post—lat A treatment. The initial shear stress—induced displacement magnitudes of fibrils after 4 h of lat A treatment were significantly reduced in comparison to those after 1 h of lat A ($0.93 \pm 0.09 \mu\text{m}$ at 1 h vs. $0.54 \pm 0.07 \mu\text{m}$ at 4 h, $n = 10$, $p < 0.001$, t-test).

DISCUSSION

Structural dynamics at a subcellular length scale reveal mechanical interactions that occur on a time scale and at spatial locations consistent with mechanochemical signal transduction. In particular, if hemodynamic forces are transmitted from the cell surface and distributed through the cytoskeleton to focal adhesion sites to initiate mechanochemical signaling, then one would expect to measure changes in relative structural dynamics from inside to outside the cell in response to onset of shear stress. This study presents the first direct measurements to demonstrate that onset of steady unidirectional shear stress altered within minutes the structural dynamics both inside and outside of the EC cytoplasm, reflecting a dynamic force environment at the cell-matrix interface. Some observations were made in both subconfluent and confluent cells; those observations include significant changes in the angle of lateral displacement of actin stress fibers and vimentin filaments, and decreased centripetal remodeling of focal adhesions and actin stress fiber termini adjacent to newly polymerized actin at cell edges. However, it is important to note that many of the shear induced changes in structural dynamics were strongly dependent upon cell plating conditions which conferred the degree of culture confluency and matrix assembly. In the cytoskeleton, onset of shear stress resulted in increased actin polymerization into lamellipodia, in both subconfluent (plated for 16 hrs) and confluent cells; however, the newly polymerized actin in confluent cells produced smaller and less stable protrusions when compared to the persistent lamellipodia formed by subconfluent cells. For the focal adhesion sites, the onset of shear stress resulted in an overall reduction of their remodeling rates in subconfluent cells, but did not significantly change the mean remodeling rate of adhesion sites measured in confluent cells. Alternatively, the onset of shear stress promoted a shift in the mean direction of adhesion displacement toward the downstream direction. Shear stress—dependent deformation of the fibronectin network in the extracellular matrix varied with the structure of the pre-existing fibril network. Onset of shear stress attenuated displacement of short fibrils (length $< 15 \mu\text{m}$) that were assembled by cells 16 h after plating. Cells grown for 48 h developed an extensive fibronectin matrix consisting of a network of fibrils that extended under multiple cells. Within one minute after onset of shear stress, fibril displacement under these confluent cell monolayers occurred primarily in the downstream direction, and this trend continued during the remainder of the 15-min flow interval.

The rapid changes in structural dynamics in the pre-existing cytoskeletal networks reflect localized mechanical strain that may serve to initiate mechanosignaling events. In cytoskeletal networks isolated from the cell, 10% stretch promotes the recruitment of multiple focal

adhesion—associated proteins to the cytoskeleton, including focal adhesion kinase (FAK), p130Cas, and paxillin, through a mechanism in which strain alters the molecular conformation of the cytoskeletal components (26). The recruitment of these proteins to the cytoskeletal network promotes the formation of new adhesion sites and adhesion signaling. Our observations of the shear stress—induced dynamic response of the cytoskeleton indicate a spatially heterogeneous strain field; displacement magnitude and direction of cytoskeletal elements is increased in some regions but reduced in other areas of the cell. It is not yet clear whether a heterogeneous structural response yields a heterogeneous biochemical response such as spatial variation in the recruitment or activation of focal adhesion proteins.

Although previous work revealed strain focusing in the intermediate filament cytoskeleton (15), little is known about the strain interaction between cytoskeletal networks. Here we show that onset of shear stress served to initiate intermediate filament displacement that closely parallels that of the microfilament network (Fig. 3). These two filament systems are linked at the molecular level through cross-linking proteins such as plectin, and plectin may in turn mediate the small GTPase regulated structural response of the actin cytoskeleton (12,18). Alternatively, if onset of shear stress induces cell spreading in subconfluent layers, then the parallel patterns of microfilament and intermediate filament displacement may simply reflect passive deformation of interconnected cytoskeletal elements and their associated membrane attachment sites. However, this model seems less likely than a decentralized hypothesis that includes active feedback from mechanosignaling pathways for two reasons. First, the time scale of cytoskeletal displacement shown here corresponds with that of regulation of Rho family proteins associated with cytoskeletal organization and contractility after onset of shear stress (32-34). Second, the spatial distribution of the displacement or strain field is not directed primarily in a radial direction with respect to the cell centroid (15), which would be expected for passive cell spreading induced by shear stress. Moreover, in confluent monolayers, onset of shear stress does not induce cell spreading, yet a heterogeneous spatial distribution of flow-induced cytoplasmic strain exists (15). In any event, it is probable that the change in lateral displacement of stress fibers redistributes the magnitude and direction of intracellular strain, but the functional implications of mechanical interactions among intermediate filaments and stress fibers remain unsolved.

The formation of lamellipodia in response to onset of shear stress involves submembrane actin dynamics and nucleation of new interactions with the extracellular matrix. Previous studies demonstrated induction of lamellipodial protrusions with the onset of shear stress in both sparsely plated cells (19) and wounded monolayers (35). We show here that this process also occurs in confluent monolayers, although the magnitude of lamellipodium extension is reduced compared to that in single cells. Stabilization of these protrusions involves the formation of new focal complexes, as detected by paxillin-DsRed2 recruitment (Fig. 2). Paxillin that is localized to new focal complexes is phosphorylated at tyrosine 118 within 8 minutes of the formation of the new complex (2). Interestingly, shear stress promotes paxillin phosphorylation in downstream regions of sparsely plated ECs while downregulating paxillin phosphorylation in upstream regions (35). The shear-induced spatial polarization of paxillin recruitment and phosphorylation preferentially to downstream lamellipodia suggests that paxillin plays a critical role in establishing directional polarity in cell migration or mechanotaxis under hemodynamic shear stress (19).

New lamellipodia induced by shear stress not only serve as new domains of paxillin recruitment but also represent local regions of Rac activation (32) that contribute to changes in cell shape and motility. In our studies, new lamellipodium formation occurred within or adjacent to regions of reduced stress fiber and focal adhesion displacement (Figs. 3 and 4). These two phenomena may result from localized activation of Rac activity and simultaneous inactivation of Rho within 5-10 min after onset of shear stress (32,33), the same time scale of our

measurements. In support of this hypothesis, Rac has been documented to antagonize Rho activity via low molecular weight protein tyrosine phosphatase (LMW-PTP) activation of p190RhoGAP (23,26).

If the regulation of Rho activity by shear stress impacts force transmission from the cytoskeleton through focal adhesion sites to the extracellular matrix, then cytoskeletal strain and associated focal adhesion dynamics would guide fibronectin assembly and remodeling. Under no-flow conditions, ECs continually remodel focal adhesions and progressively stretch fibronectin fibrils by as much as 34%, consistent with the observation that the fibronectin matrix is prestressed in vitro (24). In a response dependent upon cell plating conditions, onset of shear stress either inhibited structural focal adhesion remodeling and fibronectin strain or significantly altered the direction of adhesion site displacement and fibronectin fibril deformation. It is important to note that the observed changes in matrix deformation were dependent upon the cell layer confluency and structure of the matrix itself. When cells were plated for 16 hours, the cells resided more closely to the stiff glass substrate and assembled short rhodamine-fibronectin fibrils that underwent reduced deformation with the onset of shear stress (Fig. 9). Transient inactivation of Rho and reduced cell contractility after onset of shear stress (33) is likely to be responsible for this effect, since remodeling and assembly of the fibronectin matrix depends upon Rho activity (36). After 48 hrs of plating, a contact-inhibited confluent monolayer of cells was established, and an extensive fibrillar rhodamine-fibronectin matrix existed with multiple connections linking fibrils over the length scale of multiple cells. The onset of shear stress immediately induced a transient and predominantly downstream deformation of this interconnected matrix (Fig. 10). The initial downstream directionality of displacement of the matrix was coupled to a downstream displacement of the focal adhesion sites (Fig. 11). This uniformity in direction may reflect passive force transmission within the confluent monolayer and could generate a directional strain in the focal adhesions and matrix that has the potential to initiate spatial patterns in new integrin ligation. Stretching fibronectin by 30-35% induces increased recruitment of soluble fibronectin to the assembled fibronectin by as much as 7-fold (37), supporting the hypothesis that cell-mediated deformation of the basement membrane contributes to extracellular matrix assembly. However, it remains to be investigated whether shear stress—induced changes in fibronectin deformation can impact new fibronectin assembly.

The connectivity from the cytoskeleton through focal adhesions to the extracellular matrix regulates force transmission in a shear stress environment. The degradation of the actin cytoskeleton with lat A (100 nM, 4 h) compromised adhesive structures and yielded a reduction in the displacement of fibronectin fibrils after flow onset, perhaps due to elimination of the active contractility mediated component of traction against the ECM. The residual displacement of the fibronectin matrix may be due to transmission through some remaining actin stress fibers or may be due to passive transmission of force through and around the cell body. In combination with previous measurements of shear stress—induced strain focusing in the cytoskeleton (15), the dependence of matrix displacement on actin dynamics shown here demonstrates that the actin cytoskeleton provides a mode for the direct transmission of both external force and intracellular tension to adhesion sites and matrix. Mechanotransduction at focal adhesions and adherens junctions may in turn act as a feedback mechanism that regulates intracellular tension through Rho-mediated pathways.

Maintenance of arterial wall permeability, prevention of thrombosis, and regulation of nitric oxide production and vascular tone are the major functions of the endothelium, and all are compromised in atherosclerosis-prone regions of the vasculature that experience complex hemodynamic force profiles. Thus, elucidation of mechanisms of mechanotransmission through endothelial cells to trigger signaling networks that regulate gene expression represents a critical challenge in vascular wall physiology. Structural dynamics at a subcellular length

scale cannot be measured in vivo. However, quantitative analysis of mechanical interactions among the cytoskeleton, focal adhesion sites, and extracellular matrix in endothelial cells in vitro reveals intracellular structural cues involved in physiological cell functions relevant to sensing of directional cues in the microenvironment and establishment of directional cell migration. For example, fluorescence speckle microscopy and correlation analysis of molecular motions in migrating kidney epithelial cells suggests the existence of a “hierarchical slippage clutch” at the molecular scale that serves to regulate traction force transmission from the cytoskeleton to the substrate (17).

This hypothesis may also partially explain differential patterns of relative displacement induced by onset of shear stress acting on individual endothelial cells in a subconfluent layer (Fig. 13). Displacement of both F-actin and vinculin near stress fiber termini is suppressed after onset of shear stress (Figs. 4-6), consistent with the idea that a mechanical clutch partially couples vinculin in focal adhesion sites to F-actin stress fibers. At these locations, actin polymerization increases as new lamellae extend preferentially in the direction of shear stress. Cells in subconfluent layers appear to downregulate extracellular matrix remodeling and to stabilize adhesive interactions while simultaneously mobilizing machinery associated with establishing planar cell polarity and directional migration. Overall, unidirectional shear stress enhances the functional goal of establishing a confluent endothelial layer barrier, which is critical for wound healing or re-establishing physiological function at atherosclerosis-prone regions of the artery.

In contrast, onset of shear stress induces cells in a confluent monolayer to display transient bursts of actin polymerization near edges in random directions. Force transmitted from the cell surface causes displacement of F-actin stress fibers preferentially in the downstream direction to convey directional mechanical cues to the cell, but the displacement field is spatially heterogeneous due to local geometry of microfilament connections. Displacement of focal adhesion sites and attached fibronectin fibrils occurs in the downstream direction within one minute after onset of shear stress, but the direction of displacement becomes more heterogeneous as cells return to physiological steady-state mechanisms regulating stable adhesion to the matrix. Even in the absence of changes in displacement rate, this instantaneous directionality and its associated strain focusing (15) is expected to convey directional cues that trigger processes associated with directional structural remodeling. The functional consequences of these intracellular mechanical cues in a confluent monolayer include cell-cell junction and basement membrane remodeling to support physiological endothelial functions in atherosclerosis-resistant regions of the artery wall.

How do cells sense directionality of external cues? It is possible that acute changes in displacement direction at the subcellular scale triggers mechanosignaling mechanisms associated with directional adaptation at longer time scales. The rapid downstream displacement of both focal adhesions and fibronectin fibrils with the step onset of shear stress was a transient mechanical response that became more variable during the subsequent 15-min interval. The increased spatial heterogeneity as time progresses is likely to be due to the activation of contraction generated by the cell. Active contractile processes are not expected to be directed rapidly in the direction of shear stress, since this response requires the long-term (>24 hr) process of adaptation that is dependent upon complete structural reorganization of the cell (22). Thus, the onset of shear stress yields transient subcellular deformations in the direction of flow by a process of mechanotransmission. Importantly, these deformations are localized to subcellular regions implicated in mechanosignaling. Notably, this behavior only existed in confluent cells that resided on an extensive and interconnected fibronectin matrix but not in subconfluent cells in close contact with the stiff glass substrate. Thus, shear stress generates a transient mechanical displacement of subcellular structures that is enhanced by the presence of a distensible matrix.

The cytoskeleton, adhesions and extracellular matrix are intimately connected, and changes in the structural dynamics of one of these subcellular components have direct mechanical impact on the others. This study reveals for the first time spatial and temporal relationships in structural dynamics from inside to outside the cell in response to onset of shear stress. Dynamic regulation of mechanical strain among these structures reflects mechanical connectivity across the cell-matrix interface, but the mechanisms and consequences of these mechanical interactions remain to be elucidated. Future studies must examine the relative displacements of the cytoskeleton, adhesion sites and matrix in order to determine whether the interaction strain between these structures activates mechanosensitive signaling networks that directly mediate adaptation of the endothelium under long-term changes in the shear stress profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

New lamellipodium formation in ECs within a confluent monolayer transiently expressing EGFP- β -actin. **(A)** Images acquired at the beginning (*red*) and end (*green*) of a 7.5-min interval under no-flow conditions. Yellow indicates zero displacement. Scale bar, 10 μ m. **(B)** Images acquired just before (*red*) and 7.5 min after (*green*) onset of shear stress, 15 dyn/cm², left to right. Arrows indicate an increased number of new lamellipodia formed after onset of shear stress.

Figure 2. Shear stress-induced dynamics of EGFP- β -actin (*green*) and paxillin-DsRed2 (*red*) near the downstream edge of an EC (**A**) before and (**B**) 10 min after onset of shear stress, 15 dyn/cm², left to right. Scale bar, 5 μ m.

Figure 3.

Shear stress-induced cytoskeletal dynamics of ECs within a confluent monolayer transiently expressing both (A, C, E) EGFP-vimentin and (B, D, F) mRFP-actin. Images of the same cell were acquired just before (*red*) and 8 min after (*green*) onset of shear stress, 15 dyn/cm², left to right. Yellow indicates zero displacement. (A, B) Whole cell view. Yellow dots in (B) indicate positions of stationary red fluorescent microspheres on the coverslips that served as fiducial markers for image registration. Scale bar, 20 μm. (C-F) Magnified view of regions of interest in the same cell. Arrows indicate local direction of displacement. Scale bar, 5 μm.

Figure 4.

Centripetal remodeling of stress fiber termini containing EGFP- β -actin expressed transiently in an EC. **(A)** Images acquired at the beginning (*red*) and end (*green*) of a 10-min interval under no-flow conditions. Asterisks indicate locations of centripetal stress fiber displacement. Yellow indicates zero displacement. Scale bar, 5 μm . **(B)** Images acquired just before (*red*) and 10 min after (*green*) onset of shear stress, 15 dyn/cm^2 , left to right. Arrows indicate regions of new lamellipodium formation.

Figure 5.

Centripetal remodeling of focal adhesion sites containing paxillin-DsRed2 expressed transiently in an EC. **(A)** Images acquired at the beginning (*red*) and end (*green*) of a 10-min interval under no-flow conditions. Asterisk indicates location of centripetal focal adhesion displacement. Yellow indicates zero displacement. Scale bar, 5 μm . **(B)** Images acquired just before (*red*) and 10 min after (*green*) onset of shear stress, 15 dyn/cm^2 , left to right.

Figure 6. Cumulative displacement of representative stress fiber termini (SF) and their spatially associated focal adhesion sites (FA) illustrates reduced centripetal remodeling rates after onset of shear stress at Time = 0 min.

Figure 7.

Smoothed histogram of the displacement magnitude distribution for the population of focal adhesion sites in an EC transiently expressing EGFP-vinculin and grown on Rd-FN for 16 h at subconfluence. Displacement magnitude was computed during consecutive 15-min intervals before (*static*) and after (*flow*) onset of shear stress, 15 dyn/cm².

Figure 8.

Shear stress-induced change in the displacement direction of focal adhesion sites under a confluent monolayer of ECs expressing EGFP-vinculin. **(A)** Angular distribution of focal adhesion displacement direction during a 15-min interval under no-flow conditions. **(B)** Angular distribution of focal adhesion displacement direction within 1 min of onset of shear stress. **(C)** Angular distribution of focal adhesion displacement direction during a 15-min interval under flow conditions, 15 dyn/cm², left to right.

Figure 9.

Spatial mapping of shear stress-induced displacement distribution in the extracellular matrix underlying subconfluent cells plated for 16 h. (A) Rd-FN-labeled extracellular matrix fibrils (red) located under ECs transiently expressing EGFP-vinculin (green). Scale bar, 20 μm . (B) Spatial map of DI relative to focal adhesion positions (white) during a 15-min interval under no-flow conditions. Color scale: violet, DI = 0; red, DI = 1. (C) Spatial map of DI relative to focal adhesion positions (white) during the consecutive 15-min interval just after onset of shear stress, 15 dyn/cm^2 , left to right.

Figure 10.

Shear stress-induced change in the displacement pattern of Rd-FN fibrils in the extracellular matrix under a confluent monolayer of ECs. **(A-C)** Example overlay images from one field of view. **(A)** Images acquired at the beginning (*red*) and end (*green*) of a 15-min interval under no-flow conditions. Arrows indicate directions of fibril displacement during the intervals. Yellow indicates zero displacement. Scale bar, 10 μm . **(B)** Images acquired 1.5 min before (*red*) and 1 min after (*green*) the onset of shear stress. **(C)** Images acquired at the beginning (*red*) and end (*green*) of a 15-min interval under flow conditions, 15 dyn/cm^2 , left to right. **(D-F)** Angular distribution data from 15 fields of view. **(D)** Angular distribution of fibronectin fibril displacement direction during a 15-min interval under no-flow conditions. **(E)** Angular distribution of fibronectin fibril displacement direction within 1 min of onset of shear stress. **(F)** Angular distribution of fibronectin fibril displacement direction during a 15-min interval with shear stress, 15 dyn/cm^2 , left to right.

Figure 11.

The initial downstream directionality of displacement of the matrix is coupled to downstream displacement of the focal adhesion sites in cells within a confluent monolayer. (A) Images of a subregion of a cell expressing EGFP-vinculin acquired 1.5 min before (*red*) and 1 min after (*green*) the onset of shear stress. Arrow indicates a fluorescent microsphere used to register images. Yellow indicates zero displacement. (B) Images of Rd-FN incorporated into matrix fibrils in the same subregion featured in (A) acquired 1.5 min before (*red*) and 1 min after (*green*) the onset of shear stress, 15 dyn/cm², left to right. Scale bar, 5 μm.

Figure 12.

The displacement of the matrix after flow onset is reduced with increased exposure to latrunculin A. (A) Images of Rd-FN acquired 1.5 min before (*red*) and 1 min after (*green*) the onset of shear stress with exposure to lat A for 1 h. (B) Rd-FN under cells treated with lat A for 4 h, acquired 1.5 min before (*red*) and 1 min after (*green*) the onset of shear stress, 15 dyn/cm², left to right. Scale bar, 10 μ m.

Subconfluent

Confluent

Figure 13.

Shear stress induces the subcellular structures of ECs to undergo rapid and significant patterns of structural dynamics that vary with cell density. In subconfluent cells, flow onset increases actin polymerization at cell edges, changes the direction of lateral stress fiber (blue, dashed-blue) displacement, reduces the centripetal remodeling of actin stress fibers (blue) and adhesion sites (green), and reduces the displacement of the underlying fibronectin matrix (red). Over time, the new actin polymerization becomes polarized in the downstream direction, and new adhesion sites stabilize the cells at sites of downstream lamellipod formation. In confluent cells, flow onset generates a rapid and transient burst of edge ruffling and a transient downstream displacement of the subcellular structures. As time progresses, the displacement patterns evolve to become more heterogeneous.