Mapping the dynamics of shear stress-induced structural changes in endothelial cells

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Mott RE, Helmke BP. Mapping the dynamics of shear stress-induced structural changes in endothelial cells. Am J Physiol Cell Physiol 293: C1616–C1626, 2007. First published September 13, 2007; doi:10.1152/ajpcell.00457.2006.—Hemodynamic shear stress regulates endothelial cell biochemical processes that govern cytoskeletal contractility, focal adhesion dynamics, and extracellular matrix (ECM) assembly. Since shear stress causes rapid strain focusing at discrete locations in the cytoskeleton, we hypothesized that shear stress coordinates alters structural dynamics in the cytoskeleton, focal adhesion sites, and ECM on a time scale of minutes. Using multiwavelength four-dimensional fluorescence microscopy, we measured the displacement of rhodamine-fibronectin and green fluorescent protein-labeled actin, vimentin, paxillin, and 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 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 toward the downstream direction within 1 min after onset of shear stress. These spatially coordinated rapid changes in the structural dynamics of cytoskeleton, focal adhesions, and ECM are consistent with focusing of mechanical stress and/or strain near major sites of shear stress-mediated mechanotransduction.

mechanotransduction; cytoskeleton; extracellular matrix; focal adhesion

THE ENDOTHELIUM REGULATES a number of physiological vascular functions, such as maintenance of vessel tone, prevention of thrombosis, promotion of fibrinolysis, and initiation of atherogenesis. 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 been possible. If hemodynamic forces are transmitted through the cytoskeleton to adhesion sites, then the structural dynamics associated with cytoskeleton–focal adhesion interactions and extracellular matrix (ECM) remodeling will serve as indicators of changes in mechanical interactions.

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 re-arrangements suggest locations of mechanical stimuli of biochemical activity on the subcellular length scale. For example, 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 activity at 5 min after onset of shear stress and a decrease 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 actomyosin 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.

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The physical connection from the cytoskeleton to the ECM through focal adhesion sites may also contribute to shear stress-induced ECM remodeling. Fibronectin expression by ECs is transiently suppressed immediately following the onset of shear stress but is significantly increased after 48 h, at which time bundled fibrils 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 metalloproteinase 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 ECs 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 subconfocal and confocal EC layers the dynamic structural response of the cytoskeleton, focal adhesions, and ECM to onset of unidirectional laminar shear stress. We hypothesized that onset of shear stress results in focused force transmission, which alters within minutes the relative structural dynamics of the actin cytoskeleton, focal adhesion sites, and fibronectin matrix, and that 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 stress 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 DMEM containing 10% calf serum, 2 mM l-glutamine, 50 µg/ml penicillin G, and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were plated onto no. 1½ coverslips marked with 0.1-µm-diameter fluorescent microspheres (Invitrogen), as described previously (14). For transient transfections, we used Lipofectin (Invitrogen) to express pEGFP-actin (Clontech, Mountain View, CA) and paxillin-DsRed2 (a gift from A. F. Horwitz, University of Virginia), red fluorescent protein (mRFP)-β-actin (pCMV-mRFP-actin; a gift from E. Fuchs, Rockefeller University), and enhanced green fluorescent protein (EGFP)-vimentin (pEGFP-IvIM-Myc; a gift from R. D. Goldman, Northwestern University) or EGFP-vinculin (pEGFP/V1-1066; a gift from S. W. Craig, Johns Hopkins University) (4). Fibronectin (Sigma-Aldrich, St. Louis, MO) was labeled using the EZ-Label rhodamine protein-labeling kit (Pierce Biotechnology, Rockford, IL). To image fluorescently labeled fibronectin, we coated glass coverslips with rhodamine-fibronectin (Rd-FN) at 20 µg/ml for 30–60 min and rinsed the coverslips 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 (Bioprotechs, 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 (LatA), ECs were grown to confluence on glass coverslips coated with Rd-FN and then treated with complete growth medium containing 100 nM LatA (Calbiochem, La Jolla, CA) for 1–6 h. Treated cells were subjected to 15 dyn/cm² shear stress with medium containing 100 nM LatA or fixed with 4% paraformaldehyde. Fixed cells were stained with FITC-phalloidin (Sigma), vinculin monoclonal antibody (clone hVIN-1, Sigma), or vascular endothelial (VE)-cadherin polyclonal antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA). Affinity-purified, Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Image acquisition. Four-dimensional images were obtained with a DeltaVision restoration microscopy system (Applied Precision, Issaquah, WA) consisting of an Olympus IX70 microscope with mercury lamp illumination, a ×60/1.4 NA objective, emission wavelengths of 528 and 617 nm, and an cooled charge-coupled device 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 overexpressing fluorescent fusion proteins at moderate levels. In differential interference contrast microscopy images, cell morphology and behavior were identical in transfected and nontransfected ECs, and transfected ECs that were incorporated into confluent monolayers were indistinguishable from adjacent nontransfected 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 nontransfected 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 exported to TIFF format. Microsphere positions were used to subtract coverslip movement from the time-lapse data. Intervals before and after onset of shear stress were analyzed. 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. 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(x,y,t+τ) acquired at times t and τ, respectively, was computed as the product moment correlation coefficient (PMCC)

\[
\text{PMCC}(t,τ) = \frac{\text{Cov}[I(x,y,t), I(x,y,t+τ)]}{\sqrt{\text{Var}[I(x,y,t)] \text{Var}[I(x,y,t+τ)]}}
\]

where Cov(f) and Var(f) are the spatial covariance and variance, respectively, computed from the images. DI was computed as

\[
\text{DI}(t,τ) = 1 - \text{PMCC}(t,τ)
\]

DI values were computed for subregions of the images of the fibronectin matrix and 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 ± SE, 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 ± SE, and mean displacements before and after onset of shear stress were compared (paired t-test,
allowed rejection of the null hypothesis that correlation was considered significantly greater than zero if the -actin and paxillin-DsRed2, time-lapse imaging indicated low levels of constitutive edge activity (Fig. 1 left to right). Arrows indicate an increased number of new lamellipodia formed after onset of shear stress.

\[ t = \frac{r \sqrt{N - 2}}{\sqrt{1 - r^2}} \] (3)

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

RESULTS

Onset of shear stress induces rapid lamellipodium and focal complex formation. In subconfluent or confluent cell layers expressing EGFP-\( \beta \)-actin and paxillin-DsRed2, time-lapse image sequences were acquired at 2.5-min intervals for 10- to 15-min periods 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-\( \beta \)-actin images at the beginning (red) and end (green) of a 7.5-min period indicated low levels of constitutive edge activity (Fig. 1A). 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) with that 7.5 min after (green) onset of shear stress revealed multiple locations of significant lamellipodium activity (Fig. 1B). Many of these protrusions were not stable (see supplemental movie 1), but some persisted through the 10-min period and served to increase projected cell area by 3.8 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 at 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-\( \beta \)-actin. After 1 h of exposure to shear stress, these focal complexes matured into larger focal adhesions within stable lamellae.

Shear stress induces shifts in 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 preexisting stress fibers. 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 periods 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 the 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 in the upstream region of the cell was not different from that in the downstream region. Changes in the angle of displacement induced by onset of shear stress were as large as 180° with a mean absolute value of 100 ± 77° (\( n = 15 \) 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 supplemental movies 2 and 3).

Lateral displacements in the vimentin network are similar to those in actin stress fibers. Previous studies showed that the 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 on the order of 1 μm, we measured relative displacement between intermediate filaments and microfilaments by cotransfecting ECs with EGFP-vimentin and mRFP-\( \beta \)-actin (Fig. 3). Comparison of

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1 Supplemental information for this article is available at the American Journal of Physiology-Cell Physiology website.
images immediately 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, and E) and actin (Fig. 3, B, D, and F) cytoskeletons. Displacement of vimentin filaments closely paralleled that of actin stress fibers in direction and magnitude in subconfluent and confluent cells. Interestingly, shear stress-induced lateral displacements often correlated with the direction of the new lamellipodial protrusions (Fig. 3, C and D, arrows).

Shear stress reduces centripetal remodeling of stress fibers and their terminal focal adhesions adjacent to areas of new actin polymerization. Since onset of shear stress induced drastic 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 subpopulation 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 stress fibers containing EGFP-β-actin (Fig. 4) and focal adhesion sites containing paxillin-DsRed2 (Fig. 5). 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 the 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 ± 0.03, n = 6, P < 0.005). Interestingly, 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 ±
6% of focal adhesion sites exhibited reduced displacement after the 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 periods before (static) and after (flow) the onset of shear stress for subconfluent cells plated for 16 h and confluent cells plated for 48 h. 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 the onset of shear stress (Fig. 7). In contrast, the mean adhesion site displacement during the no-flow period in confluent cells (n = 13 cells) was not significantly different from that during the interval after the 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 ± 22°, n = 378 in 12 cells, P < 0.0001) during the 1st min after the onset of shear stress (Fig. 8B). The displacement direction again became uniform over the remainder of the 15-min period (Fig. 8C).

Shear stress induces rapid shifts in deformation of fibronectin fibrils in ECM that are associated with changes in adhesion dynamics and dependent on cell density. To determine whether shear stress-induced structural dynamics in the cytoskeleton and focal adhesion sites were transmitted to the ECM, cells expressing EGFP-vinculin were plated on coverslips coated with Rd-FN for 16 h to develop mature focal adhesions and a fibrillar fibronectin matrix. Subconfluent cells assembled the Rd-FN into short (<15-μm-long) fibrils that terminated on focal adhesions containing EGFP-vinculin (Fig. 9A). A DI of 0 (blue) to 1 (red) was computed to quantify the spatial distribution of the 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. 9B). In these subconfluent cell layers, the onset of shear stress significantly decreased DI (mean change in DI = −0.12 ± 0.02, n = 8 fields of view, P = 0.001), and DI remained reduced for ≥15 min (Fig. 9C). This pattern of reduced DI correlated with the significant decrease in mean adhesion displacement (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 period. After the onset of shear stress, fibril lengths stabilized, indicating that shear stress inhibited additional mechanical strain in the fibronectin matrix composed of short fibrils.

To measure the impact of shear stress on the fibronectin matrix underlying a confluent monolayer of cells, we plated ECs on coverslips coated with Rd-FN for 48 h, and images were acquired at 2.5-min intervals for 15-min periods before and after the onset of shear stress (see supplemental 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 period demonstrated that the fibril displacement patterns were heterogeneous (Fig. 10A) and the displacement direction was uniformly distributed (Rayleigh’s test, P = 0.11). During the 1st min after the onset of shear stress, the fibril displacement was directed primarily in the downstream direction (Fig. 10B), with a mean resultant angle of −7 ± 21° with respect to the axis parallel to shear stress (Rayleigh’s test, P < 0.0001, n = 260 fibrils in 15 fields of view). During the remainder of the 15-min flow period, this downstream directionality was reduced (mean resultant angle = −46 ± 42°) but still significant (Rayleigh’s test, P < 0.0001) as the displacement pattern of the fibrils gradually became more spatially heterogeneous (Fig. 10C). 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 (Fig. 11; see supplemental movie 5).

LatA 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 LatA, a toxin that blocks actin polymerization by binding actin monomer, for 1–6 h to the cell culture medium. 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 LatA on the actin cytoskeleton on a confluent monolayer were gradual and less visually obvious than those on subconfluent cells (unpublished observations). There were few structural differences between untreated and treated cells after 1 h, but the effects of LatA were apparent by 4 h (see supplemental Fig. 1H), but their actin-dense peripheral bundles, stress fibers, and adherens junctions remained largely intact (see supplemental Fig. 1, 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 (see supplemental Fig. 1, F, I, and L).

The direction and magnitude of fibronectin matrix fibrils were measured to determine whether LatA impacted shear stress-induced displacement. Under static conditions, the displacement directions were uniformly distributed (see supplemental Fig. 2A; Rayleigh’s test,  = 0.23). During the 1st min after the onset of shear stress, fibril displacement was directed primarily in the downstream direction (see supplemental Fig. 2B), with a mean resultant angle of ±21 ± 29.8° with respect to the axis parallel to shear stress (Rayleigh’s test,  < 0.0001, n = 304 fibrils in 8 fields of view). During the remainder of the 15-min flow period, this downstream directionality was again reduced (see supplemental Fig. 2C) but still significant (Rayleigh’s test,  < 0.0001) as the displacement pattern of the fibrils gradually became more spatially heterogeneous. Throughout the LatA treatment period, the characteristic shear stress-induced downstream displacement of the fibronectin matrix (see supplemental Fig. 2) remained similar to the directional profile measured without LatA (Fig. 10, E–G). However, the magnitude of displacement decreased with increasing duration of LatA exposure. At 1 h (Fig. 12A), 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. 12B) with increasing LatA exposure. The magnitude of displacement appeared to be dependent on fibrillar architecture, so we tested the significance of the change in displacement magnitude by subjecting the same field of view to shear at 1 and 4 h after LatA treatment. The initial shear stress-induced displacement magnitudes of fibrils after 4 h of LatA were significantly reduced compared with those after 1 h of LatA (0.93 ± 0.09 vs. 0.54 ± 0.07 μm, n = 10,  < 0.001; t-test).

DISCUSSION

Structural dynamics on a subcellular length scale reveal mechanical interactions 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 the inside to the outside of the cell in response to the onset of shear stress. This study presents the first direct measurements to demonstrate that the onset of steady unidirectional shear stress changed within minutes the structural dynamics inside and outside of the EC cytoplasm, reflecting a dynamic force environment at the cell-matrix interface. Some observations were made in subconfluent and confluent cells, e.g., significant changes in the angle of lateral displacement of actin stress fibers and vimentin filaments and decreases in centripetal remodeling of focal adhesions and actin stress fiber termini adjacent to newly polymerized actin at cell edges. However, many of the shear stress-induced changes in structural dynamics were strongly dependent on cell plating conditions, which conferred the degree of culture confluency and matrix assembly. In the cytoskeleton, the onset of shear stress increased actin polymerization into lamellipodia in subconfluent (plated for 16 h) and confluent cells; however, the newly polymerized actin in confluent cells produced protrusions that were smaller and less stable than those of 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 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 ECM varied with the structure of the preexisting fibril network. The onset of shear stress attenuated displacement of short (<15-μm-long) fibrils 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 1 min after the 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 period.

The rapid changes in structural dynamics in the preexisting cytoskeletal networks reflect localized mechanical strain, which 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, 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 are increased in some regions of the cell but reduced in others. It is not 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 the onset of shear stress initiates intermediate filament displacement, which 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 the 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. 1) The time scale of cytoskeletal displacement shown here corresponds with that of regulation of Rho family proteins associated with cytoskeletal organization and contractility after the onset of shear stress (32–34). 2) The spatial distribution of the displacement or strain field is not directed primarily in a radial direction with respect to the cell

Fig. 9. Spatial mapping of shear stress-induced displacement distribution in extracellular matrix (ECM) underlying subconfluent cells plated for 16 h. A: Rd-FN-labeled ECM fibrils (red) located under ECs transiently expressing EGFP-vinculin (green). Scale bar, 20 μm. B: spatial map of displacement index (DI) relative to focal adhesion positions (white) during 15-min no-flow period. Color scale is as follows: violet, DI = 0; red, DI = 1. C: spatial map of DI relative to focal adhesion positions (white) during consecutive 15-min periods immediately after onset of 15 dyn/cm² of shear stress (left to right).
centroid (15), which would be expected for passive cell spreading induced by shear stress. Moreover, in confluent monolayers, the 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 unresolved.

The formation of lamellipodia in response to the onset of shear stress involves submembrane actin dynamics and nucleation of new interactions with the ECM. Previous studies demonstrated induction of lamellipodial protrusions with the onset of shear stress in sparsely plated cells (19) and wounded monolayers (35). We have shown that this process also occurs in confluent monolayers, although the magnitude of lamellipodial extension is reduced compared with 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 min 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 stress-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 they 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

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Fig. 10. Shear stress-induced change in the displacement pattern of Rd-FN fibrils in ECM under a confluent monolayer of ECs. A–C: overlay images from 1 field of view. A: images acquired at the beginning (red) and end (green) of a 15-min no-flow period. 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) onset of shear stress. C: images acquired at the beginning (red) and end (green) of a 15-min of shear stress at 15 dyn/cm² (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 no-flow period. 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 15 min of 15 dyn/cm² of shear stress (left to right).

Fig. 11. Initial downstream directionality of displacement of the matrix is coupled to downstream displacement of 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 subregion shown in A acquired 1.5 min before (red) and 1 min after (green) the onset of 15 dyn/cm² of shear stress (left to right). Scale bar, 5 μm.
Rac activity and simultaneous inactivation of Rho within 5–10 min after the onset of shear stress (32, 33), the same time scale as our measurements. In support of this hypothesis, Rac has been documented to antagonize Rho activity via low-molecular-weight protein tyrosine phosphatase 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 ECM, 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 on cell plating conditions, the onset of shear stress 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 changes in matrix deformation were dependent on the cell layer confluency and the structure of the matrix. When cells were plated for 16 h, the cells adhered more closely to the stiff glass substrate and assembled short Rd-FN fibrils that underwent reduced deformation with the onset of shear stress (Fig. 9). Transient inactivation of Rho and reduced cell contractility after the onset of shear stress (33) are likely to be responsible for this effect, since remodeling and assembly of the fibronectin matrix depend on Rho activity (36). After 48 h of plating, a contact-inhibited confluent monolayer of cells was established, and an extensive fibrillar Rd-FN 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 sevenfold (37), supporting the hypothesis that cell-mediated deformation of the basement membrane contributes to ECM 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 ECM regulates force transmission in a shear stress environment. The degradation of the actin cytoskeleton with LatA (100 nM, 4 h) compromised adhesive structures and reduced the displacement of fibronectin fibrils after the onset of shear stress, perhaps because of 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 passive transmission of force through and around the cell body. In combination with previous measurements of shear stress-induced strain focusing on 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 external force and intracellular tension to adhesion sites and matrix. Mechanotransduction at focal adhesions and adherens junctions may, in turn, act as a

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**Fig. 12.** Displacement of the matrix after flow onset is reduced with increased exposure to latrunculin A (LatA). **A**: images of Rd-FN acquired 1.5 min before (red) and 1 min after (green) the onset of shear stress with exposure to LatA for 1 h. **B**: Rd-FN under cells treated with LatA for 4 h, acquired 1.5 min before (red) and 1 min after (green) the onset of 15 dyn/cm² of shear stress (left to right). Scale bar, 10 μm.

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**Fig. 13.** Shear stress induces 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 direction of lateral stress fiber (blue, dashed-blue) displacement, reduces centripetal remodeling of actin stress fibers (blue) and adhesion sites (green), and reduces 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 lamellipodium 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, displacement patterns evolve to become more heterogeneous.
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 ECs to trigger signaling networks that regulate gene expression represents a critical challenge in vascular wall physiology. Structural dynamics on a subcellular length scale cannot be measured in vivo. However, quantitative analysis of mechanical interactions among the cytoskeleton, focal adhesion sites, and ECM in ECs 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 suggest the existence of a “hierarchical slippage clutch” on 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 the onset of shear stress acting on individual ECs in a subconfluent layer (Fig. 13). Displacement of F-actin and vinculin near stress fiber termini is suppressed after the 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 ECM remodeling and stabilize adhesive interactions and simultaneously mobilize 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 reestablishing physiological function at atherosclerosis-prone regions of the artery.

In contrast, the 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 because of local geometry of microfilament connections. Displacement of focal adhesion sites and attached fibronectin fibrils occurs in the downstream direction within 1 min after the 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) are 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 on the subcellular scale trigger mechanosignaling mechanisms associated with directional adaptation on longer time scales. The rapid downstream displacement of 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 period. 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-h) process of adaptation that is dependent on 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 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 ECM 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 the inside to the outside of the cell in response to the 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 to determine whether the interaction between these structures activates mechanosensitive signaling networks that directly mediate adaptation of the endothelium under long-term changes in the shear stress profile.

REFERENCES

DYNAMICS OF SHEAR-INDUCED STRUCTURAL CHANGES IN ECs