Vascular endothelial cells (ECs) are highly specialized to respond to local changes in blood flow, particularly hemodynamic shear stress forces that act at the luminal cell surface in the direction of flow.1,2 The mechanisms responsible for endothelial mechanotransduction have physiological and pathological relevance. Acute dilatation or constriction of arteries in response to changes of flow is controlled by the endothelium through the release of nitric oxide, prostaglandins, and related molecules.3–5 Furthermore, chronic changes in local flow characteristics stimulate EC-dependent structural remodeling of the artery wall,6 and flow is implicated in the localization of atherosclerotic lesions.7 Both acute and chronic changes appear to be regulated principally by shear stress.2

ECs respond to shear stress by initiating a cascade of intracellular events that begins with the generation of second messengers and is quickly followed by transcriptional, synthetic, and structural changes (reviewed in References 2, 8, and 9). Initiation of mechanosignaling events has been measured within seconds of exposure to flow; however, the mechanism(s) by which shear stress acting on the luminal cell surface is converted to specific cellular responses is unclear. Shearing forces may directly deform the cell surface10 to generate local biochemical responses arising from undefined sensor proteins11 and/or deformation of the lipid bilayer.12 Signal transduction, however, may not be limited exclusively to the luminal surface. In a decentralization model,2 forces acting on the cell surface evoke responses at other locations within the cell, perhaps via force transmission through the cytoskeleton. Apical shear stress induces directional movement of basal focal adhesion contacts and phosphorylation of proteins concentrated at these sites.13,14 Thus, instead of operating through a single sensing pathway for mechanical forces, cellular mechanotransduction may result from an integrated response of multiple signaling networks that are spatially organized throughout the surfaces and interior of the ECs. Although extensive slow remodeling of the endothelial cytoskeleton in response to shear stress has been inferred from fixed-cell studies,1,15,16 the use of green fluorescent protein (GFP) now allows direct observation of spatiotemporal dynamics in living cells.17,18 In the present study, we have used a GFP-vimentin fusion protein19 expressed in ECs to evaluate changes in position of the intermediate filament (IF) cytoskeleton during a step change in hemodynamic shear stress.

Materials and Methods

Cell Culture and Transfection

Bovine aortic ECs were transfected for 2.5 hours with 1 to 2 μg pEGFP-hVIM-Myc19 using a liposomal method (Lipofectin, Life Technologies) according to the manufacturer’s recommendations. Cells were allowed to recover overnight in complete growth me-
Red fluorescent microspheres, 0.1 μm in diameter (Molecular Probes), were dried onto sterile No. 1.5 thickness glass coverslips (Bioptechs). Microspheres served as fiducial markers that allowed removal of coverslip movements during time-lapse image acquisition (see below). Transfected ECs were plated onto the coverslips over the microspheres and were grown to confluence.

To establish cell lines with stable expression of GFP-vimentin, ECs were maintained in growth medium containing 400 μg/mL G418 (Life Technologies) beginning 2 days after transfection. Because the plasmid contained a kanamycin/neomycin resistance gene,19 only cells expressing GFP-vimentin survived the selection pressure. Groups of cells expressing similar levels of fluorescence intensity were isolated and grown to confluence.

Deconvolution Microscopy
Wide-field fluorescence optical sections were acquired using a DeltaVision system (Applied Precision). Spatial and temporal normalization of the illumination intensity allowed quantitative analysis of GFP-vimentin fluorescence intensity.20,21 A quantitative method for image restoration improved the spatial precision of IF locations over that typically measured by wide-field microscopy. Stacks of optical sections spaced 0.1 to 0.5 μm apart were acquired. A 3D point spread function was measured experimentally, and a constrained iterative deconvolution algorithm20 was applied to arrays of optical sections. Resolution in restored images was 317 nm in the xy-plane and 758 nm along the z-axis. Maximum intensity volume projections were computed using DeltaVision software.

Time-Lapse Imaging of GFP-Vimentin in Living Cells
Coverslips containing ECs expressing GFP-vimentin were assembled into a temperature-controlled parallel plate flow chamber (FCS2, Bioptechs) and maintained at 37°C. Dual-wavelength 3D image stacks with 0.5-μm spacing between optical sections were acquired every 90 seconds for 20 to 30 minutes. A step change in flow was imposed so that wall shear stress was 12 dyn/cm², and image acquisition continued at the same rate for an additional 20 to 30 minutes. A red fluorescent microsphere on the coverslip was chosen as a fiducial marker, and its 3D position in the image stack was determined at each time point. Single fluorescence optical sections from regions of interest were chosen so that image position along the x-, y-, and z-axes was normalized relative to that of the microsphere. In this manner, motion of GFP-vimentin in the time-lapse measurements was attributable only to IF displacement and not to movement of the coverslip. Three-dimensional analysis was performed on images of 1 to 5 cells from each of 4 experiments.

Results
Visualization of GFP-Vimentin Networks in ECs
The 3D distribution of IFs in living ECs was observed using GFP-vimentin (Figure 1). Deconvolved optical sections at 2-μm intervals near the base, middle, and apex of transiently (Figure 1A) and stably (Figure 1B) transfected confluent ECs are shown in the first 3 panels of the figure. Maximum-intensity volume projections in the fourth panels were constructed from stacks of optical sections acquired at 0.1- to 0.5-μm intervals. Typically, a prominent perinuclear ring was visible, and IFs extended above and below the nucleus. A mesh network of IFs radiated throughout the cytoplasm from the perinuclear ring, and thick circumferential IF bundles were sometimes present near the cell edge or reaching into regions containing lamellipodia. In some cells, a higher concentration of GFP-vimentin–labeled IFs was associated with juxtanuclear centers. The distribution of IFs was similar after transient or stable transfection. The spatial patterns of GFP-vimentin IFs were consistent with immunocytochemically defined IF distribution in endothelium22,23 and other mammalian cells.19,24,25 Furthermore, indirect immunofluorescence labeling of vimentin with a monoclonal antibody colocalized with GFP-vimentin (data not shown), indicating that GFP-vimentin was incorporated into the preexisting endogenous vimentin network as previously demonstrated.19 The 0.1-μm red fluorescent microspheres visible in the last
panel of Figure 1B are fiducial markers on the glass surface used to normalize for coverslip displacement during flow.

**IF Motion During a Change in Shear Stress**

Time-lapse movies of deconvolved optical sections demonstrated that IFs do not form a static architecture but are dynamic in living ECs, even in the absence of externally imposed forces (see http://www.circresaha.org for online time-lapse movies). During constitutive motion, the number of filaments remained constant, and inter-IF connections were unchanged.

ECs were exposed to unidirectional laminar steady flow (wall shear stress, 12 dyn/cm²), and IF positions were compared in fluorescence optical sections from 6 time points at 3-minute intervals before (t₁, t₂, t₃) and after (t₄, t₅, t₆) a step change in flow. For consecutive time points, images from the beginning and end of the intervals were false-colored red and green, respectively. Therefore, in merged color images, yellow represented no change in GFP-vimentin position.

In contrast to the apparently random fluctuations in IF position under no-flow conditions, significant directional displacement of IFs (by nearly 1 μm) in regions of the cell occurred within 3 minutes of the onset of shear stress (Figure 2A), and increased displacement continued during exposure to flow. This was not attributable to cell migration; the position of each cell within the confluent monolayer remained unchanged throughout the observation period (up to 45 minutes). Flow-induced IF displacements were spatially heterogeneous within the same cell. Correlation plots of GFP-vimentin fluorescence intensity at each pixel for consecutive time points were measured. In such plots, larger scatter relative to the diagonal corresponded to fewer yellow pixels in the image (i.e., decreased overlap in IF positions) and increased IF displacement during the interval. The correlation coefficient, ρ, quantitatively describes the degree of overlap between red and green images. In the field of view showing an entire cell (Figure 2A), regional displacement after flow onset caused a decreased correlation during the flow-step interval that was maintained during the subsequent flow interval, demonstrating that displacement of IF position was increased coincident with and after the onset of flow.

At higher magnification, flow-induced alteration of IF motion was clearly visible (Figure 2B). Within 3 minutes of flow onset, IFs were displaced directionally, as indicated by less overlap (yellow color) in color-merged images. Furthermore, ρ was significantly decreased during the flow-step interval (t₁–t₄) compared with the preceding no-flow interval. During the succeeding interval in the continued presence of shear stress, directional motion proceeded at a slower rate, as reflected by less separation between overlapping images. The correlation coefficient only partially recovered the value computed during the no-flow interval, indicating that IFs continued to move more actively than in the absence of shear stress but less actively than after the initial onset of flow.

Further illustrations of flow-induced IF displacement are shown in Figure 3. During no-flow, most filaments appeared primarily yellow with patches of red and green, indicating random “wiggling” of individual filament bundles. During the flow-step interval, significant displacement of filaments was observed both in the same focal plane (arrowheads) and out of the plane of focus (red filaments without corresponding green positions at the end of the interval). Displacement often continued at a slower rate (a smaller distance of red-green separation) during the succeeding 3-minute flow interval.

The correlation of GFP-vimentin distribution was measured for consecutive time points before (t₁, t₂, t₃) and after (t₄, t₅, t₆) flow onset (Figure 4); ρ was computed for fields of view containing 1 to 5 cells (●, solid line) or for several subregions (□, dashed line) in which IF motion was significantly altered by flow. For consecutive no-flow intervals, ρ remained constant, indicating a steady-state constitutive motion of IFs. For fields of view containing whole cells or multiple cells, ρ decreased slightly on average at the onset of flow; however, larger and more variable decreases were computed in the subregions, reflecting subcellular heterogeneity of IF redistribution. Partial recovery of ρ in these regions during the subsequent flow intervals suggested that IFs continue to move more actively than in the absence of flow. These data demonstrate that significant initial displacement of IF occurs in response to shear stress and that distribution of flow-induced IF movement is heterogeneous within the cell.

**Spatial Measurements of IF Displacement**

To evaluate quantitatively the spatial redistribution of IFs, fluorescence intensity line profiles were obtained from optical sections. During a 3-minute no-flow interval, small IF displacements were observed in color-merged optical sections acquired at the beginning (red) and end (green) of the interval (Figure 5A). In intensity profiles measured at the beginning (red) and end (green) of the no-flow interval (Figure 5B), most peaks did not change intensity or position (arrows), indicating zero displacement of the corresponding filaments along the x-axis. Several peaks were displaced along the optical axis, as indicated by a change in intensity at constant x-axis position (open arrow). In contrast, red-green filament pairs and fewer yellow pixels showed that some IFs were significantly displaced during the succeeding flow-step interval (Figure 5C). Comparison of intensity profiles before and after flow onset (Figure 5D) confirmed that some IFs were significantly displaced along the flow axis (arrowhead) whereas others moved into or out of the focal plane (open arrows), as indicated by a change in peak intensity without displacement along the x-axis. A few peaks did not change intensity or average x-position (arrows), corresponding to zero displacement. Thus, filaments only a few micrometers apart exhibited different displacement responses to the onset of shear stress.

IF network motion was analyzed in 3 dimensions (Figure 6). Nodes connecting a minimum of 3 filaments were chosen from 34 randomly selected positions in 5 cells selected from 3 confluent monolayers. The positions of the nodes projected on the x-, y-, and z-axes were tracked relative to reference positions (x₀, y₀, z₀) just before flow onset (time=0 minutes). Step movements in the z-direction were larger than those in the xy-plane due to lower resolution along the optical axis. Before the start of flow, movement consisted of fluctuations around a constant average position along the x-, y-, and z-axes (time=0 minutes). After the onset of flow along the x-axis (time>0 minutes), a number of nodes underwent significant...
Figure 2. Change in IF motion due to onset of shear stress (flow direction, left to right; shear stress, 12 dyn/cm²). Fluorescence optical sections were compared at consecutive time points under no-flow conditions (t₂, t₃), before (t₃) and after (t₄) a step increase in flow, and with flow (t₄, t₅) applied to the cells. Images from the beginning and end of the 3-minute intervals were false-colored red and green.
directional movement. These nodes moved rapidly during the first 3 minutes of flow and then continued to fluctuate around a new average position. Nodes throughout the cytoplasm moved in various magnitudes and directions, but mean relative positions of nodes located higher in the cell were more likely to be affected by flow than nodes near the basal surface. Furthermore, the change in IF distribution measured after flow onset was primarily due to initial displacement rather than altered spatial fluctuations of IF position. Thus, the variability in IF positions increased significantly, suggesting rapid force redistribution through the cell within 3 minutes of the onset of shear stress.

Discussion

These experiments represent the first spatiotemporal measurements of cytoskeletal motion in response to a controlled mechanical environment with physiological relevance (flow). The decentralization model of mechanotransduction\textsuperscript{2} proposes that the dynamic response of the cytoskeleton to an instantaneous change in the distribution of extracellular applied forces plays a key role in the integration of fast signaling responses. The early changes in IF displacement observed in response to shear stress suggest that 3D force redistribution throughout the cell acts rapidly at sites micrometers away from the luminal surface.

ECs contain an extensive interconnecting cytoplasmic network of vimentin IFs. Several physiological roles for IFs have been proposed,\textsuperscript{26} including determination and maintenance of cell shape, transmission of mechanical stress, targeting of molecules between the nucleus and cytoplasm, and regulation of nuclear position and morphology. The network has traditionally been regarded as a static architecture because of the low solubility and detergent extractability of IFs. However, recent studies have revealed a dynamic IF structure during cell spreading\textsuperscript{24} and division\textsuperscript{26} that depends to some extent on the state of vimentin phosphorylation.\textsuperscript{27} These properties of overall network stability combined with spatial dynamics make IFs an interesting candidate for cytoskeletal force responses.

The flexing and bending behavior of IFs without flow was similar to IF motion reported by other investigators.\textsuperscript{19,24,25} Thin IFs reaching into the cytoplasm flexed more than thicker filament bundles and organizing centers, network nodes respectively. In merged color images, yellow represented zero displacement during the interval. In correlation plots of fluorescence intensity at each pixel for consecutive time points, larger scatter relative to the diagonal corresponded to fewer yellow pixels in the image (increased IF displacement during the interval). Correlation coefficient, $r$, measures degree of overlap between red and green images; $r=1$ for perfect overlap (no movement), and $r$ decreases for less overlap (increased IF movement). A, Transfected cell in confluent monolayer (surrounded by nonexpressing cells). Regional displacement after flow onset caused a decreased correlation. Bar=10 μm. B, Higher-magnification view of a region near the apex of a cell. Significant displacement of IFs was measured within 3 minutes of flow onset, and slower directional movement continued in the presence of flow, demonstrated by a decrease and then partial recovery of $r$. Bar=2 μm.
moved in apparently random directions, and the interconnecting filament segments flexed or changed length accordingly. GFP-vimentin IFs maintained their connections so that assembly or disassembly of network segments was not observed as individual mesh units changed shape during these relatively short time intervals.

A complicated 3D distribution of IF movement was frequently observed. For example, IF unidirectional movement in one focal plane was accompanied by variable direction of motion in a plane lower in the cell. Fung and Liu28 have proposed that shear stresses are transmitted over the cell surface to junctions and basal adhesion sites, largely bypassing the cytoskeleton. Although this mechanism may contribute to force redistribution, our studies support a broader interpretation of force transmission through transcellular cytoskeletal displacement.

The distribution of initial network strain in response to applied shear stress may be related to IF physical properties, which are distinctly different from those of microfilament and microtubule networks.29 Polymerized vimentin in vitro has higher elasticity and a lower shear modulus than actin. However, vimentin hardens at high strain without breaking, unlike microtubules. In the present studies, disassembly and reassembly of IF network connections were not observed during the short periods analyzed. This IF network stability may be important during reorganization of microfilament and/or microtubule networks by a mechanism that requires breaking of those network connections. The combined strain-hardening and elastic properties of the vimentin IF network may therefore confer mechanical stability on the cell. Vimentin knockout mice provide further evidence for the role of IFs in mechanical stability. Although these mice grow to maturity and lack an obvious phenotype,30 a more careful evaluation has revealed regional defects at the cellular level that suggest disruption of cellular functions that depend on an intact mechanical organization.31,32 Cell-wide force distribution and mechanical properties depend on a composite material description of IFs, microfilaments, and microtubules. Thus, interactions between IFs and other cytoskeletal elements will play a role in determining the dynamic response to shear stress.33,34 Potential crossbridge elements include nestin35 and a plectin-like 300-kDa protein.23,36,37 Rapid changes in IF distribution near the cell base may also participate in a mechanism whereby the dynamics of focal adhesion sites are altered by shear stress.38 Furthermore, because the perinu-

Figure 5. Quantitative comparison of local IF movement located 1.0 μm above the coverslip during no-flow (left) and flow-step (right) intervals. A and C, Fluorescence optical sections. Flow direction in panel C was left to right with shear stress of 12 dyn/cm². Bar=5 μm. B, Comparison of fluorescence intensity line profiles measured along the x-axis in the center of the inset strip in panel A demonstrates minor IF movements under no-flow conditions. Overlapping intensity peaks indicate zero displacement of individual filaments during the 3-minute interval (arrows), whereas changes in intensity without change in peak position indicate displacement out of the focal plane (open arrow). D, Increased IF displacement during the flow-step interval. Change in peak position with constant intensity illustrates that some IFs are displaced in the x-direction by almost 1 μm (arrowhead), whereas changes in the peak intensity values indicate IF movement into or out of the focal plane (open arrows). A few IFs do not change average x-position (arrows). Flow direction was along the x-axis with shear stress of 12 dyn/cm².

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clear ring of vimentin IFs may be directly or indirectly linked to the nuclear lamina, force redistribution under flow may also affect the karyoskeleton, consistent with other mechanical perturbations. Through interactions between nuclear IF proteins, the nuclear lamins, DNA, and histones, changes in gene expression may be directly mediated by flow.

In summary, both qualitative and quantitative spatial analyses in living ECs revealed rapid regional IF displacement in response to shear stress. Although such measurements do not exclude a role for local deformation in mechanical signaling, they suggest an integrated mechanism of mechanotransduction in which spatial organization of multiple structural and signaling networks regulates cellular responses to an altered hemodynamic environment.

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References


Rapid Displacement of Vimentin Intermediate Filaments in Living Endothelial Cells Exposed to Flow

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