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Keywords

shear stress, green fluorescent protein, vimentin, intermediate filaments, decentralization model, mechanotransduction

Comments

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The Cytoskeleton Under External Fluid Mechanical Forces: Hemodynamic Forces Acting on the Endothelium

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Abstract—The endothelium, a single layer of cells that lines all blood vessels, is the focus of intense interest in biomechanics because it is the principal recipient of hemodynamic shear stress. In arteries, shear stress has been demonstrated to regulate both acute vasoregulation and chronic adaptive vessel remodeling and is strongly implicated in the localization of atherosclerotic lesions. Thus, endothelial biomechanics and the associated mechanotransduction of shear stress are of great importance in vascular physiology and pathology. Here we discuss the important role of the cytoskeleton in a decentralization model of endothelial mechanotransduction. In particular, recent studies of four-dimensional cytoskeletal motion in living cells under external fluid mechanical forces are summarized together with new data on the spatial distribution of cytoskeletal strain. These quantitative studies strongly support the decentralized distribution of luminally imposed forces throughout the endothelial cell.

INTRODUCTION

The endothelium of the arterial side of the circulation is continuously exposed to flow. Spatial and temporal variations in the associated hemodynamic shear stresses create a complex force environment at the interface between the luminal cell surface and blood plasma. These relationships represent an intriguing model of externally imposed fluid mechanical forces in a highly polarized environment.

A large number of endothelial mechanotransduction responses to shear stress have been documented.[11,15,18,54,89] However, the cell sensor(s) responsible for converting frictional shear forces into multiple specific signaling responses is unknown. Although the initial interaction between the force and the cell occurs at the luminal surface, intracellular elements that connect to that surface provide continuity of structure and mechanics that extend to other parts of the cell.[15,50,103] Therefore, mechanotransduction sites are not necessarily restricted to the cell surface. In fact, there is ample evidence that, in addition to the cell surface, the cell junctions, the nucleus, and focal adhesions are highly responsive to luminal shear stress. The central ''connector'' of these sites is the cytoskeleton. Here we summarize recent work that demonstrates spatial and temporal displacement of the intermediate filament (IF) network in living endothelial cells in response to imposed shear stress. The studies support a mechanotransmission (and possibly transduction) role for these relatively stable cytoskeletal elements. Their connection through linker molecules to transmembrane proteins at various locations throughout the cell and the identification of such sites as signaling locations in mechanotransduction suggest that the cytoskeleton is a key component of endothelial mechanotransduction, a role consistent with decentralized transmission of intracellular forces.

SPATIAL CONSIDERATIONS OF MECHANOTRANSDUCTION: THE DECENTRALIZATION MODEL

The wealth of signal transduction events induced by flow at various intracellular locations in endothelial cells does not support the view that a single mechanosensor is responsible for converting the external fluid mechanical forces into electrical, biochemical, and genetic responses. Instead, the cell can be viewed as a structure with highly organized, nonhomogeneous mechanical properties that distribute the forces internally as well as peripherally. As a result, the observed cellular responses, and ultimately adaptation, occur as a result of spatial integration of force transmission and molecular signaling events. This is the basis for a ''decentralization model'' of endothelial

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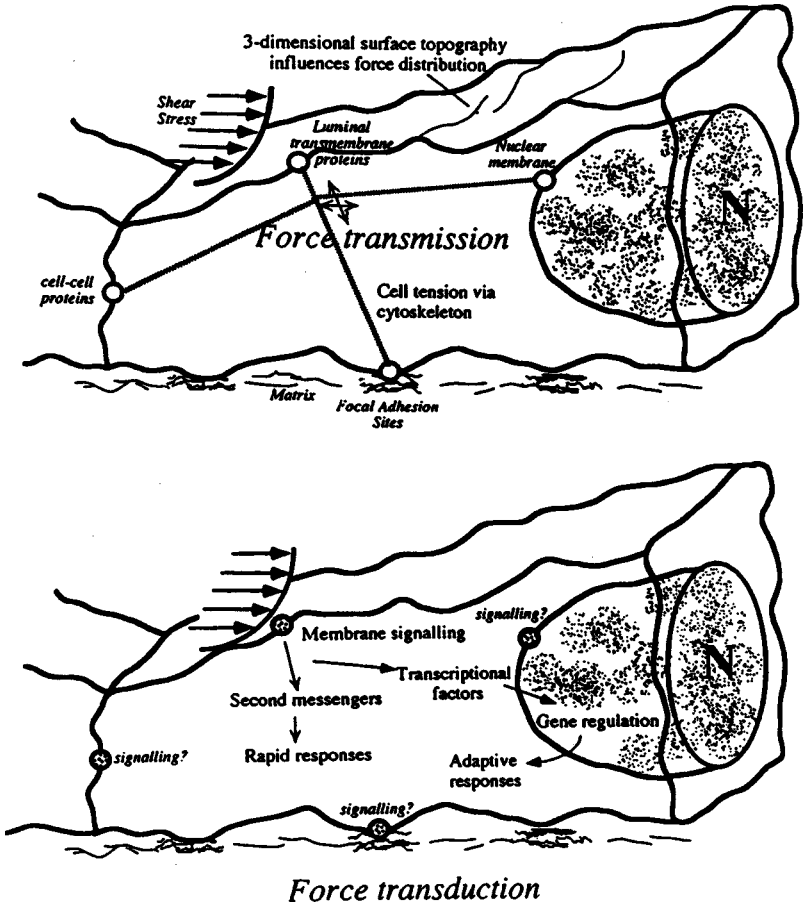

FIGURE 1. The decentralization model of mechanotransduction. Extracellular fluid mechanical forces acting on the luminal surface are transmitted by the cytoskeleton to remote intracellular locations such as cell–cell junctions, focal adhesion sites, and the nucleus. From Ref. 15 by permission.

mechanotransduction[15] as outlined in Fig. 1. Although many sites in the cell can be involved in mechanotransduction, the elements that integrate force transmission throughout the cell most comprehensively are cytoskeletal. Before considering the cytoskeleton in force transmission, however, we first outline several locations that directly interact with cytoskeletal elements to provide a continuum of structure from the luminal surface to the lateral cell junctions, the nucleus, and the basal adhesion sites. Experimental evidence suggests that signaling events at each location are involved in endothelial mechanotransduction.

The Luminal Cell Surface

The luminal surface of endothelial cells is in direct contact with the flowing blood and represents the surface of action of fluid mechanical shear and normal (pressure) forces. At a subcellular length scale, fluid shear stress magnitude and gradients vary significantly with the local surface topography,[5] thereby enhancing fluctuations in average hemodynamic forces measured in the artery lumen. Thus, the location of a flow sensor along the cell surface will affect its exposure to applied fluid forces.

Several pieces of evidence suggest that fluid forces may act directly on flow sensors at the cell surface. Activation of an inward-rectifying potassium channel occurs within seconds of altered shear stress.[78,82] as demonstrated by single channel recordings from the luminal membrane.[57] The resulting rapid hyperpolarization is followed by an extended depolarization that has been linked to activation of a chloride-selective membrane current.[4] Stretch-activated[66] or other cation[75] channels may also be activated directly by hemodynamic force acting at the cell surface. In addition to ion channels, transmembrane receptor proteins may be activated directly by fluid shear stress. For example, fetal liver kinase-1 (Flk-1), a receptor tyrosine kinase that binds vascular endothelial growth factor, is tyrosine phosphorylated and associates with signaling molecule complexes within minutes of shear stress onset.[11] The biophysical properties of the endothelial surface glycoprotein layer, or glycocalyx, may modulate the ability of these transmembrane receptors or ion channels to sense the fluid mechanical environment.[86] Physical properties of the plasma membrane itself may be altered by shear stress, leading to activation of nearby G-proteins within seconds.[38,39,41] Furthermore, signal transduction may be localized to membrane regions such

as caveolae that are both rich in signaling molecules and exhibit structure that is capable of transmitting tension.[90] Spatial localization of signaling molecules that associate with actin stress fibers also implicates caveolae in directly transmitting force to the cytoskeleton.[29]

In addition to activating cell surface molecules, extracellular forces are transmitted to the intracellular space through direct links to the submembrane cytoskeleton. Microspheres coated with ligands to integrin receptors bind to the cell surface, and resistance to optical[13,25] or magnetic[103] forces applied to the beads demonstrates direct connection to an intracellular mechanical structure. Furthermore, the cytoskeleton quickly remodels to reinforce the strength of attachment[13] through a mechanism that involves activation of signaling molecules.[26] These observations demonstrate a close relationship between locally applied force, cytoskeletal dynamics, and signaling pathways near the cell surface. It is likely that similar pathways are involved in flow-mediated mechanotransduction.

Cell–Cell Junctions

The surfaces between adjacent cells experience shear force as a result of torque on the cell body by hemodynamic shear stress, and normal forces may result as cells are compressed by elevations in blood pressure. These intercellular surfaces contain junctional complexes that regulate barrier function and vasoconducted responses. Both communication[19] and structural[80] junctions adapt their spatial distribution in response to altered hemodynamic -shear stress. Proteins located in adherens junctions, including VE-cadherin, α-catenin, β-catenin, and plakoglobin, localize to the ends of actin stress fibers following adaptation to shear stress,[80] suggesting that monolayer integrity is maintained by mechanical connections from cell to cell. Although the role of junctions in the initial signaling responses to altered hemodynamic forces remains unclear, it is likely that adherens junctions act as a mechanical link between the cytoskeletal networks of neighboring cells.

The Abluminal Surface

The basal (abluminal) surface of endothelial cells experiences a complicated mechanical profile. In addition to tension and torque resulting from hemodynamic forces applied to the cell, the cell itself generates traction forces on its substrate during migration and differentiation. Focal adhesion complexes rich in both structural and signaling molecules[6,7] mediate the transmission of force from the substrate to the cytoskeleton. Several molecular signaling events result, including integrin clustering and phosphorylation of focal adhesion kinase and paxillin.[11,16,53,70] In addition, the focal adhesion complexes appear to function as structural scaffolds for the recruitment of cytoplasmic signaling molecules,[11,70] leading to activation of the mitogen-activated protein kinase (MAPK) signaling pathway.[53,70,98] These events parallel those that have been characterized for integrin-mediated cell adhesion.[6,7]

Focal adhesion remodeling and turnover occurs in most anchorage-dependent cell types, including endothelium. Following onset of unidirectional laminar shear stress, focal adhesion sites align their shape parallel to the flow direction without changing their overall contact area, and the rate of remodeling is related to the magnitude of shear stress.[17,106] It is not clear, however, whether this response originates from cytoskeletal tension transmitted from the luminal surface to integrins in focal adhesion complexes (inside-out signaling) or directly from altered tension between integrins and the extracellular matrix at adhesion sites (outside-in signaling). In either case, intracellular signaling pathways that regulate cytoskeletal organization are activated locally near focal adhesion complexes.[69,87,88]

Spatial Organization in the Cytoplasm

Intracellular signaling events in response to flow forces also occur at locations other than near the plasma membrane, supporting the hypothesis of spatial organization and integration in the cytoplasm. For example, fast imaging has demonstrated local intracellular calcium release and wave propagation through stimulated cells,[55,56,96,104] and the amplitude of calcium transients may depend on an intact structure of the actin cytoskeleton.[108] A similar phenomenon is expected in mechanically stimulated endothelial cells,[48,83] consistent with a hypothesis for cytoskeleton-mediated spatial organization of signal propagation in response to luminal shear stress.

Metabolic signaling pathways are also spatially organized in the cytoplasm. For example, altered shear stress or simulated ischemia induces generation of reactive oxygen species.[105] Mechanotransduction may be initiated acutely by mitochondrial sensing[2] (P. T. Schumacker, personal communication) or more slowly by adaptation in the expression of copper/zinc-containing superoxide dismutase, a cytosolic enzyme involved in the generation of reactive oxygen species outside the mitochondria.[52] Furthermore, since activation of endothelial nitric oxide synthase (eNOS) to generate nitric oxide occurs when tyrosine phosphorylated eNOS is dissociated from caveolin,[27,32,33,61] it is likely that nitric oxide generation is spatially localized in the cytoplasm near caveolae. These examples demonstrate that force-mediated regulation of signaling occurs in a manner consistent with intracellular spatial organization of signaling networks. An important and central component of this model is the cytoskeleton.

CENTRAL ROLE OF THE CYTOSKELETON IN DECENTRALIZED MECHANOTRANSDUCTION

Endothelial cells contain three major cytoskeletal networks composed of actin microfilaments, vimentin IFs, and tubulin microtubules. In addition to determining mechanical properties, the cytoskeleton in anchorage-dependent cells determines cell shape and mediates growth and differentiation[49] through spatially organized signaling mechanisms that respond to extracellular cues (reviewed in Ref. 58). The cytoskeleton also serves as the major contributor to intracellular tension that is necessary for cell adhesion and migration on the extracellular matrix.[50] Since the cytoskeleton can interact with signaling pathways that regulate its own dynamics, a state of altered tension may signal a need for cellular adaptation to the new mechanical environment.[51] Furthermore, crosstalk among signaling pathways leads to multiple cellular responses.

Among the earliest reports on the responses of cells to fluid flow forces were cell alignment[20,79] and a corresponding adaptation of cytoskeletal filament distribution.[20] Interactions among cytoskeletal filaments[59,93,102] provide continuity of structure from the luminal plasma membrane[63,103] to focal adhesion complexes.[6,7] Compelling evidence for connection of the cytoskeleton to nuclear components[40,74] also suggests a direct link between force transmission and regulation of events in the nucleus. Taken together, these observations demonstrate that the cytoskeletal structure must play a pivotal role in decentralized transmission of hemodynamic force from the luminal surface to other sites in the cell.

Although interactions among cytoskeletal networks and signaling responses to an altered hemodynamic profile have been described,[72] the coordinated mechanisms by which cells sense their mechanical environment and initiate adaptation remain to be elucidated. Changes in shear stress may have an indirect effect by inducing physically discontinuous molecular signaling events that lead to altered cytoskeletal dynamics and remodeling. However, a more direct mechanism is the transmission of fluid mechanical stresses by the cytoskeleton to redistribute intracellular force and thereby provide spatial specificity to the resulting signaling events. If this latter hypothesis is correct, then rapid displacement of cytoskeletal filaments should occur, i.e., cytoskeletal network deformation and displacement indicates a change in the spatial distribution of tension. Such observations would strongly support the decentralization hypothesis of mechanotransduction, since cytoskeletal displacement putatively mediates interactions among signaling molecules by controlling their spatial proximity. In addition, force transmitted via the cytoskeleton to signaling molecules such as ion channels, phospholipids, and integrins may affect molecular conformation, thereby activating a related signaling pathway.

LIVE CELL IMAGING OF CYTOSKELETAL DYNAMICS

Cytoskeletal Filament Dynamics Revealed by Green Fluorescent Protein

Although slow remodeling of the cytoskeleton in response to altered fluid shear stress has been documented,[20,31,72] measurements in fixed cells do not reflect the acute dynamics of the structural networks. Observation of gene expression or molecular distribution in living cells in real time has increased dramatically with the cloning of cDNA for green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*[85] and its expression in heterologous tissues.[10] In recent years, GFP has been expressed as a reporter gene, cell marker, and fusion tag[9,99] in mammalian cells and animals, plants, yeast, slime molds, fruit flies, worms, fungi,[14] bacteria,[76] and other microorganisms.[24] Since expression of GFP in mammalian cells does not significantly affect proliferation rate or other important cellular functions,[37] it serves as an endogenous fluorescent marker for spatial distribution or biochemistry.

GFP fused to cytoskeletal proteins has provided new insights into the real time dynamics of cell structure.[71] Microtubules have been observed in living cells during differentiation,[36] cell motility,[64] and cell division.[8,73] Expression of GFP-actin has revealed structural and assembly dynamics in *Dictyostelium discoideum*,[107] fission yeast,[21] *Drosophila melanogaster*,[100] and mammalian cells[3,28,81,91] with minimal effects on physiological cell function.[12] Finally, the role of IFs, whose functions are poorly understood, is under investigation using GFP-vimentin.[43,47,77,109] Thus, it is now possible to address hypotheses for the involvement of all three cytoskeletal filament networks in intracellular force transmission.[42,44]

GFP-Vimentin

IF structure and dynamics have been characterized (reviewed in Ref. 45), and putative functions in cell structure and mechanics have been proposed.[34] Vimentin IFs interact with both the microfilament[94] and microtubule[47,97] networks. Thus, a composite material description more closely describes mechanical properties of the cytoplasm than one composed of a single cytoskeletal protein.[59] Mice lacking vimentin exhibit impaired wound healing[22] and flow-induced arterial remodeling[92] that may be related to defects in contractility and mechanical integrity at the cellular level.[23] These observations suggest that vimentin IFs play an important role in the cellular response to fluid mechanical forces *in vivo*.

The GFP-vimentin fusion protein has revealed new details about the dynamics of IFs. Highly motile GFP-vimentin squiggles or dots near cell edges suggest rapid assembly and turnover in migrating or spreading cells.[77,109] These fragments colocalize with kinesin motors[84] and depend on interactions with tubulin;[47] therefore, the assembly of the IF network near cell edges depends on transport and mechanical interactions with microtubules. In the more central cytoplasmic regions of the cell, GFP-vimentin distributes to the endogenous network of IFs, as demonstrated by colocalization with antivimentin antibody staining,[109] and the steady state dynamics of GFP-vimentin turnover is equivalent to that measured with microinjected rhodamine-conjugated vimentin.[101,109] The ratio of GFP-vimentin to vimentin is typically 1:5,[47] so that the expression level of the fusion protein is relatively low compared to that of endogenous vimentin. Thus, expression of GFP-vimentin does not affect its function within the IF cytoskeleton, and GFP-vimentin can be used as a tool to visualize IFs in living cells in real time.

HIGH-RESOLUTION FOUR-DIMENSIONAL FLUORESCENCE MEASUREMENTS

To test the hypothesis that vimentin IFs in endothelial cells are deformed by fluid shear stress, high-resolution four-dimensional (4D) fluorescence microscopy followed by image restoration was performed on endothelial monolayers expressing GFP-vimentin.[43] Wide-field fluorescence optical sections were acquired (DeltaVision, Applied Precision, Issaquah, WA) using a highly corrected 63X/1.4NA objective lens (Zeiss, Germany) and a 12-bit cooled charge coupled device camera (MicroMax, Princeton Instruments, Trenton, NJ). A high precision mechanical *XYZ* stage allowed oversampling of light intensity in three-dimensions (3D); optical sections were spaced 0.10–0.25 μm apart with 0.01% accuracy. GFP was excited by a mercury arc; bandpass barrier filters were chosen so that excitation and emission wavelengths were 490 and 528 nm, respectively. Spatial and temporal normalization of the illumination field allowed quantitative analysis of fluorescence intensity.[46,62]

Quantitative image restoration improved the spatial precision over that typically measured by either conventional wide-field fluorescence or confocal microscopy.[35] Using *SoftWoRx* software (Applied Precision), three-dimensional (3D) fluorescence data were corrected along the time axis to normalize for small fluctuations in integrated fluorescence intensity. A 3D point spread function was measured experimentally, and a constrained iterative deconvolution algorithm was applied to arrays of optical sections.[46] Resolution in restored images during flow chamber experiments was typically $<$300 nm in the *xy* plane and $<$750 nm along the *z* axis. Maximum intensity volume projections were computed using *SoftWoRx*.

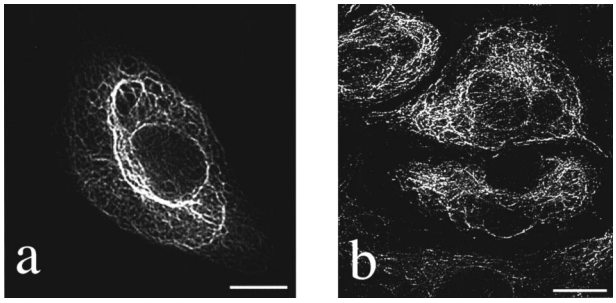

FIGURE 2. GFP-vimentin-labeled IFs in a confluent monolayer of endothelial cells (a) after transient transfection and (b) in a cell line selected for stable expression of the fusion protein. Note that adjacent cells in (a) are not expressing GFP-vimentin. Scale bar, 10 μm. From Ref. 43 by permission.

Coverslips containing confluent monolayers of endothelial cells expressing GFP-vimentin were assembled into a temperature-controlled parallel plate flow chamber (FCS2, Bioptechs, Butler, PA) and maintained at 37 °C. Red fluorescent microspheres attached to the coverslip under the cell monolayers and used as fiducial reference markers were visualized using a rhodamine filter set (λ_{ex} = 555 nm, λ_{em} = 617 nm). Dual wavelength 3D image stacks were acquired every 90–180 s for 20–30 min. A step change in flow was imposed so that wall shear stress imposed on the endothelium was 12 dyn cm^{-2}, and image acquisition continued at the same rate for an additional 20–30 min. Stationary red fluorescent microspheres were chosen as fiducial markers of coverslip position, and the 3D positions of GFP fluorescence volumes was normalized to those of the microspheres. In this manner, motion of GFP-vimentin in the time-lapse measurements represented IF movement only and not systematic movement of the coverslip. These methods allowed quantitative 4D analysis of IF distribution during changes in fluid shear stress forces, as detailed later.

CYTOSKELETAL DISPLACEMENT DURING CHANGES OF SHEAR STRESS

GFP-vimentin expressed in bovine aortic endothelial cells labeled the endogenous IF cytoskeletal network [Fig. 2(a)]. IF bundles formed an interconnected mesh throughout the cytoplasm, and thicker filament bundles were often observed near the nucleus. Transient transfection using a liposomal method[43] induced expression of GFP-vimentin in approximately 10% of cells. As a result, individual cells expressing GFP-vimentin were frequently surrounded by nonexpressing cells, allowing measurements on single cells within the monolayer. Alternatively, a cell line with stable expression of GFP-vimentin was established to allow analysis of IF motion

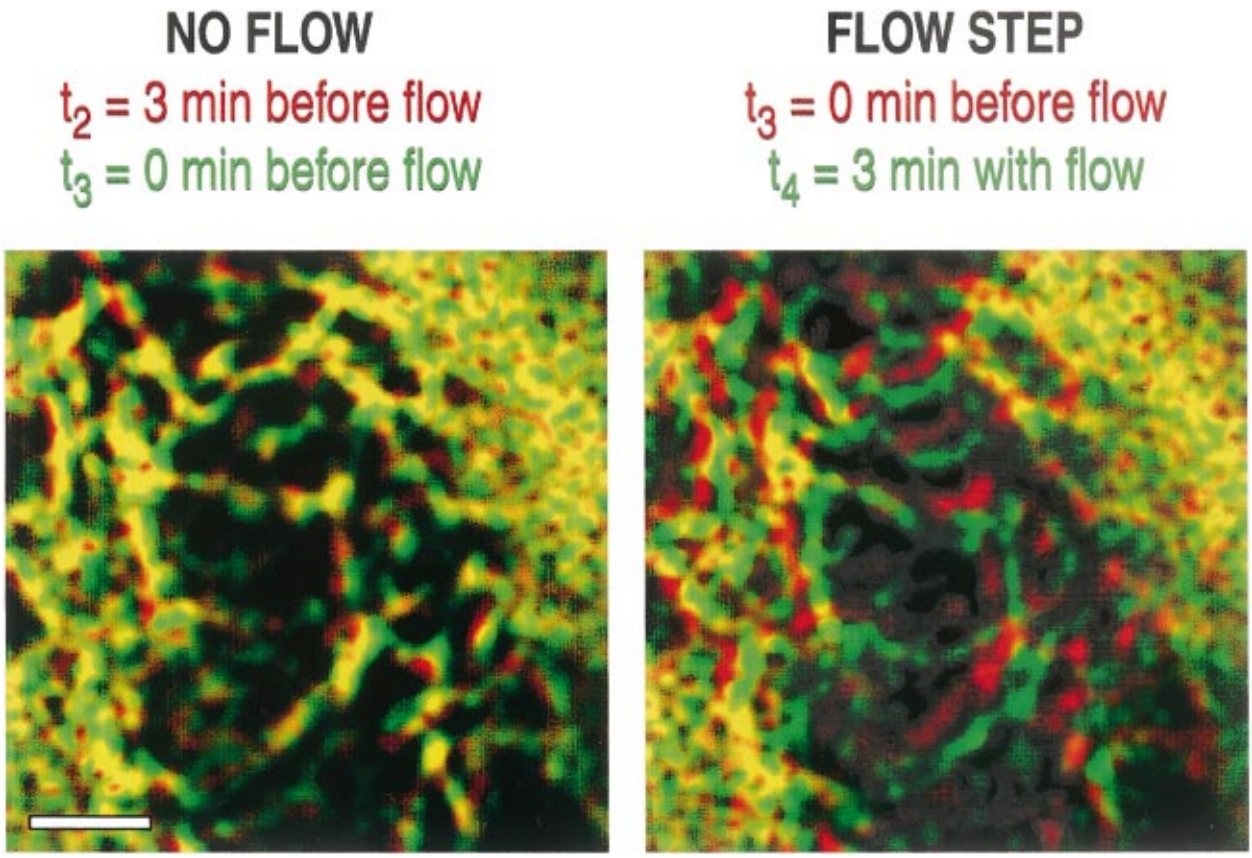

FIGURE 3. High-magnification view of IF displacement during consecutive 3 min intervals (a) without flow and (b) just after flow onset, with flow direction left to right and shear stress 12 dyn cm^{-2}. Merged color images show GFP-vimentin-labeled IFs at the beginning (red) and end (green) of the intervals; yellow represents zero displacement. Scale bar, 2 μm. From Ref. 43 by permission.

in adjacent cells simultaneously[43,44] [Fig. 2(b)]. In all cases, cell and IF morphology were not affected by expression of GFP-vimentin.

Constitutive motion of GFP-labeled IFs was observed even in the absence of flow[43,47,77,109] [Fig. 3(a)]. In a confluent monolayer, GFP-vimentin fragments were not observed near cell edges,[77,109] suggesting that the IF network had reached steady state assembly. Within minutes of flow onset, however, significant directional displacement of IF segments was superimposed on constitutive motion in localized regions of the cell [Fig. 3(b)]. Flow-induced displacement varied in magnitude and direction and was not clearly related to the flow direction. The motion of individual filaments often consisted of both translation and bending or flexing. In some regions, however, groups of filaments were translated as a rigid body without mesh deformation. IF displacement occurred in 3D at various heights above the coverslip. These observations demonstrated that onset of shear stress induced heterogeneous displacement and deformation of the IF network.

Analysis of time-lapse movies allowed direct measurement of displacement using endogenous morphological features.[43] Tracking of the 3D positions of connections among IF segments as a function of time clearly demonstrated that flow onset induced a range of displacement patterns. Before flow onset, most network features displayed random fluctuations around a constant average position. However, some segment connections were translated by as much as 1 μm within 3 min of flow onset and then maintained their new average position. Other connection points exhibited slower directional motion with approximately constant velocity. Since the patterns of displacement induced by flow onset varied, it is likely that the distribution of cytoskeletal deformation depended to some extent on the local network morphology and therefore reflected local cytoplasmic mechanical properties.

SPATIAL ANALYSES OF INTRACELLULAR DISPLACEMENT

Since time-lapse movies demonstrated heterogeneous IF motion during changes in fluid shear stress, a quantitative method was developed to measure the spatial distribution of IF displacement at a subcellular length

scale.[44] The 4D fluorescence distribution function $f(x,y,z,t)$ represented IF positions in space and time. The discrete Fourier power spectrum was analyzed to determine the spatial frequency of IF separation, and subimages were chosen so that the subsequent spatiotemporal analysis represented IF segment displacement.

The degree of overlap of fluorescence intensity between two consecutive time points represents the average magnitude of IF displacement within the spatial region of interest. The displacement index $\mathrm{DI}(t_i,t_j)$ was computed based on the spatial product moment cross correlation between two times t_i and t_j.[44] DI is invariant with respect to the absolute intensity scale and the number of nonzero data points; therefore, it represents a normalized measure of the displacement magnitude of GFP-labeled IFs within the region of interest. The computed values of DI were mapped spatially and with respect to time, revealing both spatial and temporal patterns of flow-induced IF displacement.

During no-flow intervals, small values of DI were computed in most spatial regions of cells. However, DI was significantly increased in regions of the cell during the 3 min interval following onset of shear stress (Fig. 4). In nine cells studied, a consistent spatial pattern emerged (Fig. 5). On average, DI increased with height in the cell and in spatial regions downstream from the nucleus; however, DI was unchanged in subregions below the nucleus. Larger IF displacement may be expected near the luminal surface where shear forces act directly, and smaller displacement below the nucleus may represent relative structural stability in this area of the cell. Increased IF displacement with distance along the flow axis reveals a dependence on filament connections throughout the cytoplasm, suggesting that cytoskeletal network morphology plays a role in transmitting force through the cell away from the upstream luminal surface. Furthermore, the heterogeneous spatial distribution of DI at all heights in the cell suggests that cellular deformation is not simple shear but a more complex displacement pattern that depends on mechanical interactions among cytoskeletal elements. After the initial 3 min period of exposure to shear stress, DI was decreased and reached a new steady state average rate of displacement. The motion of IFs continued to be more variable than before flow onset.

This spatiotemporal analysis of DI provides the first 3D analysis of cytoskeletal displacement in living endothelial cells under physiologically relevant levels of shear stress. Quantitative objective comparisons can be made between adjacent cells or among cells experiencing different hemodynamic conditions. Since detection of IF movement along the optical axis is possible, the 3D measurements often revealed IF displacement that was not visible in two-dimensional (2D) projection images. The DI reported IF movement on a subcellular length scale, allowing measurement of intracellular displacement that is often not visible at a whole-cell length scale. This may explain why previous studies labeling the cytoskeleton of fixed cells after exposure to shear stress were unable to detect adaptation until hours after flow onset.[20,31,68,72] Thus, measurements using DI serve as the

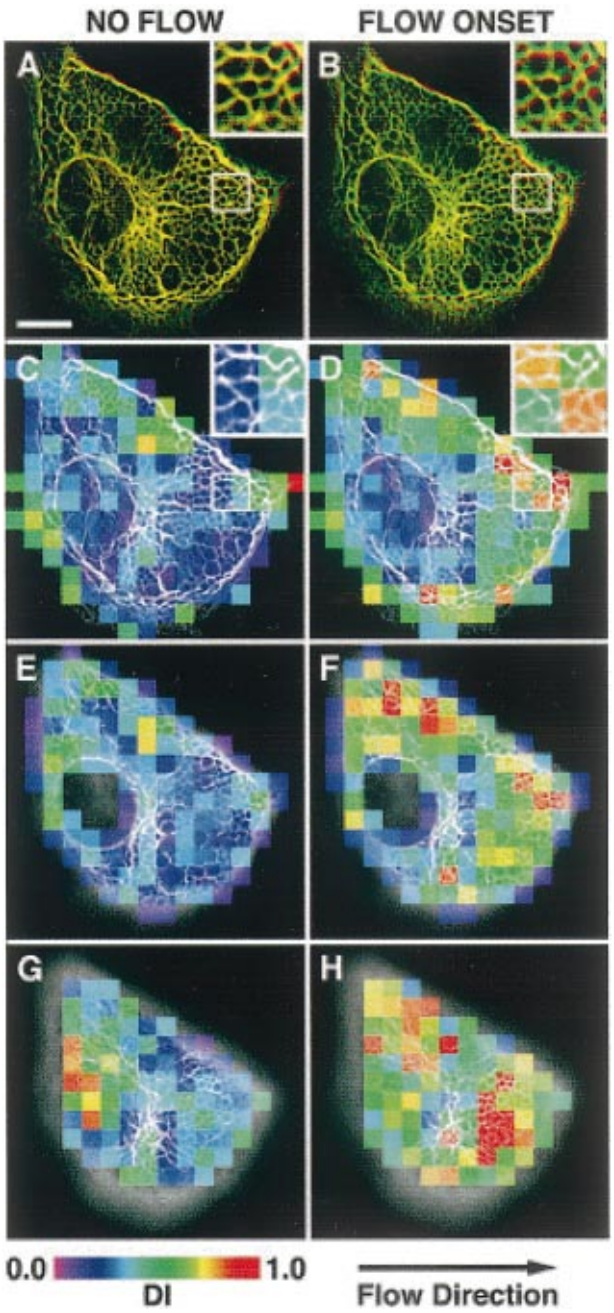

FIGURE 4. DI distribution in a single cell grouped by height for consecutive 3 min intervals with no flow, and immediately after flow onset. DI in individual 3D subimages increased in magnitude and variability just after flow onset; values correlated with height in the cell. Decreased variation was computed with continued flow. From Ref. 44 by permission.

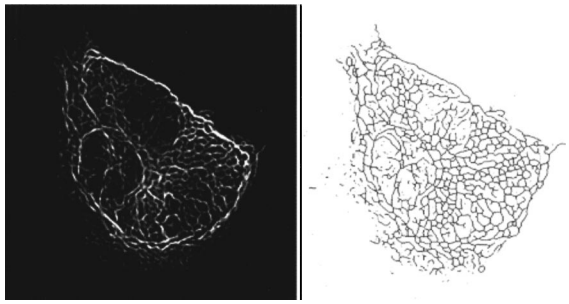

FIGURE 5. A fluorescence optical section and the corresponding skeletonized representation of the IF network.

first quantitative analysis that directly relates extracellular fluid forces to intracellular cytoskeletal displacement.

COMPUTATION OF INTRACELLULAR STRAIN FIELDS

Spatial analysis of intracellular displacement demonstrates a rapid response to altered shear stress; however, DI measures both deformation and translation. In order to detect relative deformation of adjacent IF segments, a method has been developed to compute the intracellular strain field (B. P. Helmke *et al.*, manuscript submitted). Using image processing techniques, images of optical sections were skeletonized to represent in 2D the positions of IFs (Fig. 6). Coordinates of vertices, or connection points, among three or more IF segments were extracted at each time point, and a particle tracking algorithm determined the path of each vertex as a function of time. Thus, the projected displacement field could be computed using image features extracted directly from the cytoskeleton morphology.

The Lagrangian strain tensor E_{ij} was computed from the following equation:

$$ds^2 - ds_0^2 = 2E_{ij}da_i da_j, \tag{1}$$

where ds and ds_0 are the final and initial distances, respectively, between adjacent vertices of interest, da_i is the projected initial distance onto the ith axis, and the Einstein summation convention is implied. Since the strain tensor is symmetric, Equation (1) has three unknowns and can be solved using distances measured from three adjacent line segments drawn between vertices. A computational geometry technique, Delauney triangulation, was employed to draw the smallest possible set of triangles connecting all vertices tracked in the data set. Thus, the strain tensor computed in Eq. (1) described the average strain field in the spatial regions among every set of three vertices. To find the magnitudes and directions of principal strain at each spatial location, eigenvectors were computed to diagonalize the strain tensor. The principal stretch ratios $\lambda_{I,II}$ were computed as

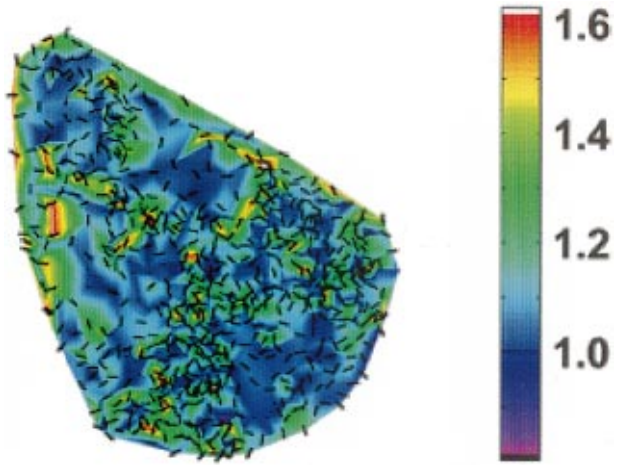

FIGURE 6. Magnitude and direction of the principal stretch ratio λ_I during the 3 min interval immediately following flow onset. Colorscale value indicates stretch magnitude, and bars indicate orientation of the stretch axis. Spatial map corresponds to the image and skeleton in Fig. 5.

$$E_{I,II} = \frac{1}{2}(\lambda_{I,II}^2 - 1), \tag{2}$$

where $E_{I,II}$ are the principal values of strain in the I and II directions, respectively.

The magnitude and direction of λ_I were analyzed graphically (Fig. 6). Localized regions of finite stretch were computed. Although areas of finite stretch were distributed throughout the cell, high strain was frequently located near upstream edges with respect to the flow direction. The principal direction of stretch in these upstream edge regions was often parallel to the flow direction, suggesting that shear stress was transmitted to the IF network in these regions as elongation forces.

The spatial map of strain during the 3 min interval following flow onset is heterogeneous and does not correlate to the map of DI discussed earlier. DI measures the degree of IF segment displacement, while the strain map shows local deformation of the IF network. Thus, the strain map illustrates regions of the cytoplasm in which adjacent IF segments were displaced either by different magnitudes or in different directions; a rigid body displacement is represented as zero strain. These data demonstrate that the onset of flow acting at the luminal surface of the endothelium induces a complex distribution of displacement that depends on local network morphology and mechanical properties; it is not a simple shear deformation of a homogeneous viscoelastic medium. Furthermore, it is likely that cytoskeletal strain indicates force redistribution through the cytoplasm.

MECHANICAL PROPERTIES IN FORCE TRANSMISSION

The high degree of heterogeneity indicated by the spatial distributions of cytoskeletal displacement and strain may result from local variations in mechanical properties, thereby contributing to spatial gradients of intracellular force. If cellular mechanotransduction depends on force transmission to discrete sites where signaling can occur, then structural mechanics in the cell plays an important role in regulating physiological processes such as endothelial barrier permeability, transendothelial transport, and regulation of vasomotor tone. These cellular biomechanical properties are determined primarily by local cytoskeletal structure.

The three individual cytoskeletal network components exhibit distinct properties measured *in vitro*.[59] F-actin and tubulin gels have shear moduli of 823 and 34 Pa, respectively. Both shear moduli are approximately constant, but these gel structures rupture at large strains. In contrast, vimentin gels show strain-hardening behavior and do not rupture over the range of strains tested, up to 80%. These behaviors suggest functional roles for each polymer network. F-actin microfilaments serve as tension bearing elements[103] that resist the greatest amount of intracellular stress at small strains.[59] At larger strains, however, both the microfilament and microtubule networks rupture,[42,59] while vimentin IFs retain their connected structure[59] (B. P. Helmke, manuscript submitted). Thus, IFs may serve to maintain the mechanical integrity of the cell during adaptation of the other two networks to an altered extracellular force environment.

The most accurate description of cellular biomechanics, however, may be that of a composite material that includes characteristics of all three cytoskeletal networks. For example, *in vitro* measurements have demonstrated that a copolymer gel of F-actin and vimentin exhibits strain hardening behavior but at higher values of shear modulus than a pure vimentin gel.[60] Although structural interactions among cytoskeletal elements have been demonstrated directly by electron microscopy[93] and indirectly by mechanical probing,[102] direct observations identifying relevant molecular interactions in living cells under physiological mechanical conditions remain to be obtained.

The cytoskeleton is connected to the nucleus via interactions between vimentin IFs and nuclear lamins.[34] As a result, fluid mechanical forces may be transmitted to the nuclear karyoskeleton, as has been demonstrated with other mechanical perturbations.[42,74] However, the motion of the nucleus during changes in fluid shear stress appears consistent with rigid body motion in a viscoelastic cytoplasm, and significant deformation of the nucleus under fluid mechanical forces has not been measured (B. P. Helmke, unpublished observations). Nevertheless, nuclear motion, heterogeneous cytoskeletal deformation,[43,44] and interactions among IFs, lamins, DNA, and histones[58] suggest that the nucleus does experience spatial force gradients that play a direct role in regulating gene expression.

Taken together, these data suggest that cellular biomechanics should be characterized by a composite in which each component makes a contribution to the overall behavior of the cell. Previous models have represented the cell as a continuous viscoelastic medium bounded by an elastic shell.[30] However, in order to explain spatial specificity of signaling that results from altered hemodynamic forces acting at the luminal surface, molecular mechanisms that depend on interactions among cytoskeletal elements and signaling molecules must be investigated.

MOLECULAR MECHANISMS OF FORCE TRANSMISSION AND TRANSDUCTION

Although the exact mechanisms of mechanotransduction remain unclear, several hypotheses based on current evidence for molecular interactions can be proposed. As discussed previously, fluid forces act directly on transmembrane proteins or the lipid bilayer itself. In addition, the plasma membrane contains structures that are capable of both transmitting force to the cytoskeleton and transducing force into biochemical signals. For example, caveolae putatively play a role in modulating membrane tension,[90] and these membrane invaginations are rich in signaling molecules, including some that interact with actin stress fibers.[29] However, mechanisms that regulate the structural and signaling links between caveolae and the cytoskeleton are unknown.

In addition to caveolae, apical plaques composed of $\alpha_5\beta_1$ integrin, vinculin, talin, and paxillin are structurally linked to the cytoskeleton.[63] It is reasonable to hypothesize that initiation of signaling may occur near these complexes in a manner similar to that described at focal adhesion sites. Paxillin phosphorylation and integrin clustering occurs within minutes after onset of shear stress,[11,53,70] and focal adhesion-associated molecules serve as a scaffold for recruitment of signaling molecules such as Grb2 and Shc[11] that play a role in activating MAPK signaling.[70] However, the critical molecular event that transduces altered tension into phosphorylation has not been elucidated.

Intracellular force transmission depends on interaction among cytoskeletal networks. Vimentin interacts with F-actin and phospholipids to regulate polymerization.[94] Vimentin transport to sites of IF network formation requires intact microtubule tracks and depends on the motor protein kinesin.[47,84] In stable structures, IF are crosslinked to microfilaments and microtubules by linker proteins, plectin[97] and nestin.[95] Multiple signaling networks involving small GTPases can regulate this balance

of cytoskeletal interactions, emphasizing the relationship between force transmission and cytoplasmic biochemical pathways.

Several molecular interactions suggest a role for intercellular junctions in mediating mechanotransduction. Adherens junctions, composed of VE-cadherin, α- and β-catenin, and plakoglobin, adapt their structure concurrently with the actin cytoskeleton. As actin dense peripheral bands dissociate during the first several hours after onset of unidirectional steady laminar shear stress, the distributions of VE-cadherin, α-catenin, and β-catenin change from continuous structures along cell edges to punctate complexes that are located only in areas of contact between adjacent cells. With continued exposure to shear stress, junctional complexes elongate into small dashes and associate with the ends of F-actin stress fibers as they assemble.[80] In some endothelial cell types, vimentin IFs are also inserted into adherens junctions through association with desmoplakin,[67] a molecule that associates with plakoglobin in epithelial junctions. Since IFs maintain their network structure and are displaced by similar direction and magnitude in adjacent cells after onset of shear stress,[43,44] this cytoskeletal component probably plays a role in the structural integrity of the monolayer by maintaining the associations among VE-cadherin, α-catenin, and β-catenin as F-actin dissociates and reassembles in response to the extracellular force. Temporary translocation of plakoglobin away from the junctions to the nucleus during adaptation to shear stress may serve to regulate gene expression by interacting with TCF-type transcription factors.[80] Interestingly, β-catenin also signals to these factors downstream of Wnt-1 activation in cardiomyocytes.[1] In these cells, β-catenin is the effector molecule for Wnt-mediated regulation of connexin-43 expression, thereby modulating cell–cell communication via gap junctions. Both connexin-43 and cadherins are associated with the Triton-insoluble cytoskeletal cell fractions,[65] and shear stress induces redistribution of connexin-43 that affects cell–cell communication.[19] These findings suggest that shear stress transmitted from the luminal surface to cell–cell junctions via the cytoskeleton regulates a complex molecular signaling network that modulates endothelial barrier permeability and intercellular communication.

These molecular interactions proposed for force transmission and transduction suggest that flow induces biochemical signal initiation at discrete sites in the cell in response to an altered intracellular force distribution. The overall cellular response is integrated, as suggested by the decentralization hypothesis, but the key molecular events that transduce mechanical force into a biochemical reaction remain unknown.

FUTURE DIRECTIONS

The availability of fluorescent cytoskeleton fusion proteins to visualize filament location in living cells is a powerful tool for spatiotemporal studies of intracellular mechanics. GFP-actin, -tubulin, and -vimentin are readily available and, together with increasingly sophisticated optical systems and advances in fluorescence resonance energy transfer and associated techniques, are leading to ever more detailed studies of the cytoskeleton and its relationships to other cellular components. Although quantitative spatial studies of filament movement can be readily performed, as illustrated here, interpretation of the biological significance is limited by the lack of detailed knowledge of the material properties of the filaments themselves. This is attributable in part to the complex organization of filamentous bundles and the dynamic reorganization that some components, actin in particular, undergo over short intervals. Perhaps more difficult to determine, however, are the properties of critical linking molecules between the cytoskeleton and its anchoring points near cell membranes. From an engineering perspective, little is known about these molecules. Furthermore, the interplay between different cytoskeletal elements that connect at nodal locations through such linkers is an added complication. As some of these gaps in knowledge are filled, an accurate mechanochemical model of the cell that reflects its complexity will become clearer.

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