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Integrin and Growth Factor Receptor Crosstalk

Mechanical Signaling and the Cellular Response to Extracellular Matrix in Angiogenesis and Cardiovascular Physiology

David Cheresh, Guest Editor

Mechanical Signaling and the Cellular Response to Extracellular Matrix in Angiogenesis and Cardiovascular Physiology

Donald E. Ingber

Abstract—Great advances have been made in the identification of the soluble angiogenic factors, insoluble extracellular matrix (ECM) molecules, and receptor signaling pathways that mediate control of angiogenesis—the growth of blood capillaries. This review focuses on work that explores how endothelial cells integrate these chemical signals with mechanical cues from their local tissue microenvironment so as to produce functional capillary networks that exhibit specialized form as well as function. These studies have revealed that ECM governs whether an endothelial cell will switch between growth, differentiation, motility, or apoptosis programs in response to a soluble stimulus based on its ability to mechanically resist cell tractional forces and thereby produce cell and cytoskeletal distortion. Transmembrane integrin receptors play a key role in this mechanochemical transduction process because they both organize a cytoskeletal signaling complex within the focal adhesion and preferentially focus mechanical forces on this site. Molecular filaments within the internal cytoskeleton—microfilaments, microtubules, and intermediate filaments—also contribute to the cell's structural and functional response to mechanical stress through their role as discrete support elements within a tensegrity-stabilized cytoskeletal array. Importantly, a similar form of mechanical control also has been shown to be involved in the regulation of contractility in vascular smooth muscle cells and cardiac myocytes. Thus, the mechanism by which cells perform mechanochemical transduction and the implications of these findings for morphogenetic control are discussed in the wider context of vascular development and cardiovascular physiology. **(*Circ Res*. 2002;91:877-887.)**

Key Words: integrin ■ cytoskeleton ■ mechanotransduction ■ tensegrity ■ capillary morphogenesis

Enormous progress has been made in terms of identifying the soluble growth factors, insoluble extracellular matrix (ECM) molecules, and membrane receptor signaling pathways that mediate angiogenesis—the growth of new blood capillaries. However, the challenge for the future is to understand how all these networks of interactions function in the physical context of living cells and tissues. For example, we tend to think of tumor angiogenesis in a linear way in which angiogenic factors produced by cancers activate growth signaling cascades in endothelial cells that lead to progressive growth and elongation of the new vessels that feed the tumor. In fact, this process is much more complex in that neighboring regions of the same growing endothelium can simultaneously exhibit very different behaviors. Careful microscopic analysis performed in the early part of the last century clearly demonstrated that growing, regressing, and quiescent differentiated capillary sprouts all coexist within the same angiogenic microenvironment.[1]

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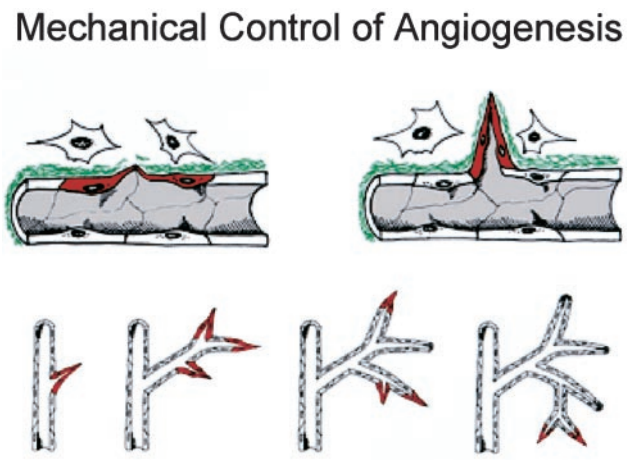

Figure 1. Mechanical control of angiogenesis. This schematic diagram visualizes how local growth differentials may drive normal tissue patterning during capillary morphogenesis. Note that migration and growth are constrained to small groups of cells (red) that are underlined by thinned regions of the basement membrane (green) due to accelerated rates of ECM turnover. Outward branching results when red cells extend and grow because neighboring cells along the same basement membrane remain quiescent (white cells).Lower magnification view shows how reiteration of this simple building rule over time and space results in creation of a complex branching capillary network with characteristic fractal-like forms. Our working hypothesis for mechanical control of angiogenesis is based on the assumption that the local thinning in the ECM produced by accelerated ECM turnover will increase ECM compliance and result in local cell distortion through the action of tractional forces exerted by surrounding cells. Increased tension transfer across cell-surface ECM receptors (integrins) will result in coordinated changes in cell and cytoskeletal form and thereby produce changes in cellular biochemistry that result in the localized growth and motility that drive capillary patterning.

The reality is that in all developing tissues, individual cells sense multiple simultaneous inputs. For example, the same time a growing vascular endothelial cell or smooth muscle cell binds a soluble mitogen, it also may form new adhesions to insoluble ECM molecules and to neighboring cells. Through these adhesions, the cell also will experience physical distortion of the tissue by hemodynamic forces or by contraction of other cells that form the developing vasculature. Yet, any individual cell only produces one concerted response: it either grows or differentiates or moves or dies. In fact, this ability of subsets of cells within the same tissue to locally exhibit differential responses to soluble stimuli underlies pattern formation in the vasculature (Figure 1) as well as in all other developing organs.[2,3] But how could this work in a tissue microenvironment that is likely saturated with soluble mitogens and motility factors?

Hypothesis

About 20 years ago, we proposed the unconventional idea that this process by which cell responses to soluble hormones are controlled locally during morphogenesis may be regulated mechanically.[2,3] Specifically, the concept was that the local variations in ECM remodeling that are observed during morphogenesis will change ECM structure and mechanics (Figure 1). For example, it was shown in the late 1970s that the local regions of basement membrane at tips of new capillary sprouts[4] and growing epithelial glands[5] become thinner due to high ECM turnover. As in any material, a thinner basement membrane should become more compliant and stretch out more than the neighboring tissue, much like a run in a woman's stocking, because all soft tissues experience isometric tension or a tensile "prestress" as a result of tractional (contractile) forces that are actively generated within their constituent cells. This decrease in ECM stiffness would change the balance of forces that are transferred across cell surface adhesion receptors that link the ECM to the internal supporting framework of the cell, the cytoskeleton (CSK). Thus, in this model, cells adherent to local regions of the ECM that thin and extend would experience increased tension on their adhesion receptors. This, in turn, would promote cell and CSK distortion and thereby alter cellular biochemistry, resulting in the localized growth and motility that drive tissue patterning. Reiteration of this process along the sides of newly formed capillary vessels would then result in formation of branching patterns that are characteristic of all growing vascular networks (Figure 1).

Experimental Insight Into Mechanical Control of Cell Behavior

Essentially, we have been able to show that ECM exerts this form of mechanical control over capillary endothelial cell behavior.[6–16] Perhaps the clearest results have come from use of a microfabrication method that allowed us to make cell distortion an independent variable. We accomplished this by adapting a micropatterning technology that was first developed by George Whitesides' Laboratory (Department of Chemistry and Chemical Biology, Harvard University, Boston, Mass) as an alternative method for creating microchips for the computer industry.[10,17] Using this technique, we were able to fabricate micron-sized adhesive islands coated with a saturating density of immobilized ECM molecules, surrounded by barrier regions that contained polyethylene glycol which prevents protein adsorption and hence are nonadhesive.

When capillary endothelial cells were cultured on circular islands coated with fibronectin (FN) that ranged in diameter from 10 to 50 μm, they spread to the limits of the island and thus took on circular shapes (Figure 2). When the same cells were attached to square islands, they became square in shape and displayed 90° corners.[10] Importantly, when cells were cultured in chemically defined medium containing a saturating amount of recombinant angiogenic factor (basic fibroblast growth factor [FGF]) and plated on different-sized adhesive islands coated with a high FN density, we found that altering cell shape produced profound effects on cell behavior. Specifically, cell growth increased in parallel as island size was raised and cell spreading was promoted,[10] even though both small and large fibronectin islands can induce similar levels of integrin signaling.[18] When island area was decreased and cell growth was shut off, apoptosis—the cellular "suicide" program—was switched on even though the cells remained adherent to the FN substrate.[10] Similar studies were also performed with substrates that contained smaller (3 to 5 μm diameter) adhesive islands that permitted cells to extend over multiple islands while maintaining the total cell-ECM contact

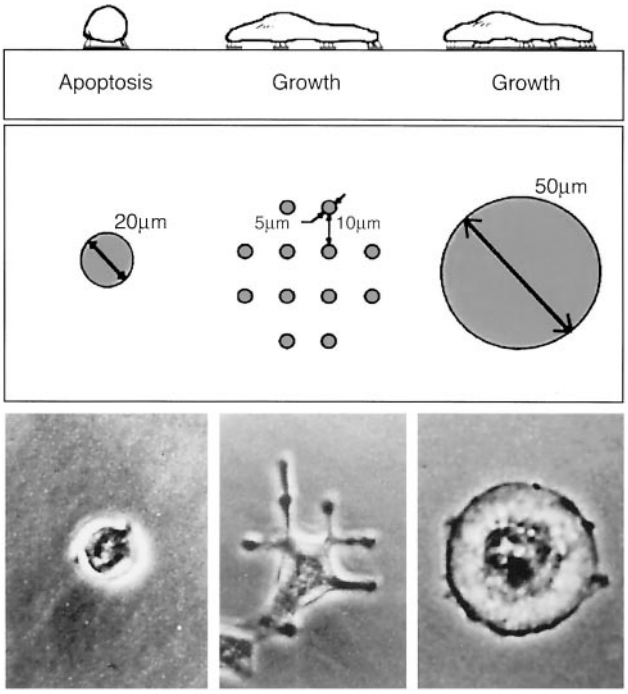

Figure 2. Cell distortion-dependent control of cell growth and apoptosis. Schematic diagram showing the initial micropattern design containing different-sized circular adhesive islands (top) and phase contrast microscopic views (bottom) of the final shapes of capillary endothelial cells adherent to surfaces fabricated with these patterns (for more details[10]).

area similar to that of one small island (Figure 2); these cells proliferated, confirming that cell distortion per se is the critical governor of cell growth versus death.[10]

Subsequent studies using linear substrates that restrained capillary endothelial spreading to a moderate degree (which neither promotes growth nor apoptosis) resulted in activation of a differentiation program, as indicated by formation of quiescent capillary tubes containing a central lumen in the same growth factor–containing medium[13] (Figures 3A and 3B). Similar induction of differentiation has been observed in vascular smooth muscle cells and primary rat hepatocytes on substrates that promote a moderate degree of spreading, as measured by quantitating contractility[19] and secretion of blood proteins,[20,21] respectively. In many of these studies, various ECM molecules (eg, FN, laminin, vitronectin, types I and IV collagen) that utilize different types of integrin receptors displayed the same effects on cell shape and function when immobilized under similar conditions.[10,12,19–21]

More recently, we extended these studies to demonstrate distortion-dependent control over directional migration in capillary endothelial cells, fibroblasts, and muscle cells.[15] Specifically, we found that if a motility factor, such as platelet-derived growth factor (PDGF) or FGF, is added to a cell adherent to a square ECM island, the cell will extend out lamellipodia, filopodia, and microspikes that drive cell migration. However, formation of these new cell processes is physically constrained in that they preferentially extend from the corners of the square cells (Figure 3C). In other words, the geometry of the substrate dictates the directionality of cell movement.

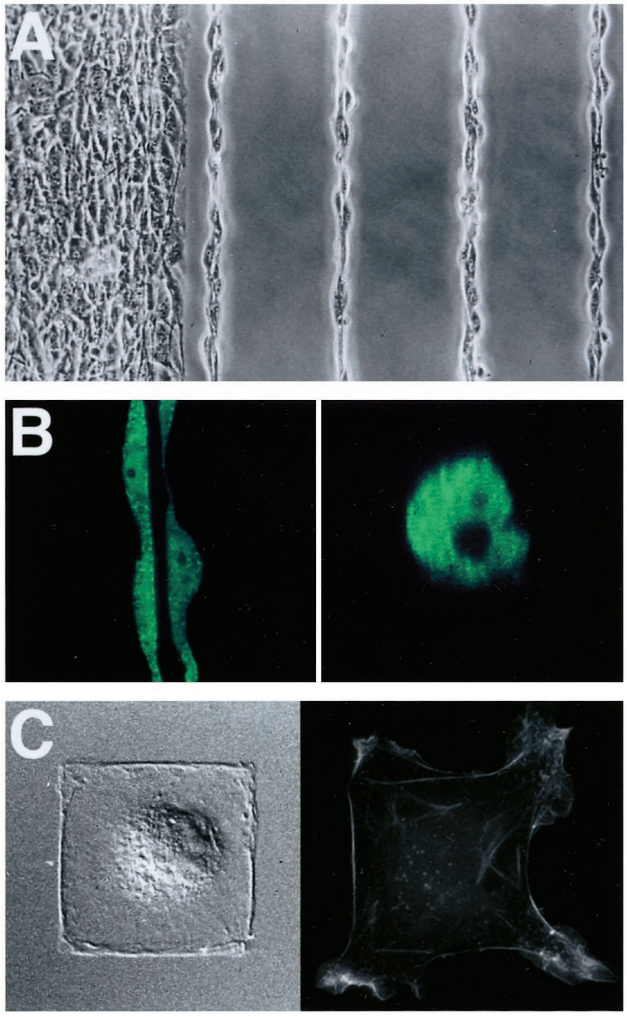

Figure 3. Capillary tube formation on linear adhesive islands (A and B) and directional extension of motile processes on square islands (C). A, Phase-contrast micrograph of capillary endothelial cells cultured for 72 hours on an unpatterned region (left) or 10-μm lines (right) coated with the same density of fibronectin ($\times$200). Note that capillary tube formation is limited to the linear islands. B, Confocal microscopic images of fluorescein-stained cells cultured on a 10-μm line showing a central cavity extending along several cell lengths when viewed in a horizontal (left; 1000$\times$) or vertical cross section (right; 3000$\times$) (for more details[13]). C, Nomarski (left) and fluorescent (right) microscopic images of cells on square (40$\times$40 μm) fibronectin islands. Left, Unstimulated cell. Right, Cell fixed 30 minutes after addition of a motility factor (FGF) and stained with fluorescent phalloidin to visualize F-actin within lamellipodia and filopodia, which preferentially extend from the corners of the square cell (for more details[15]).

As described, this ability to establish local differentials in cell growth, differentiation, apoptosis, and motility is key to morphogenesis in all developing tissues. Importantly, our in vitro studies were able to mimic this local control over endothelial cell responses to soluble stimuli that is observed during pattern formation in vivo by simply varying the degree to which cells could spread over ECM.[8–15] These data, combined with our results on vascular smooth muscle cell contractility, suggest that local cell distortion-dependent switching between growth, differentiation, apoptosis, and motility in an environment saturated with soluble mitogens

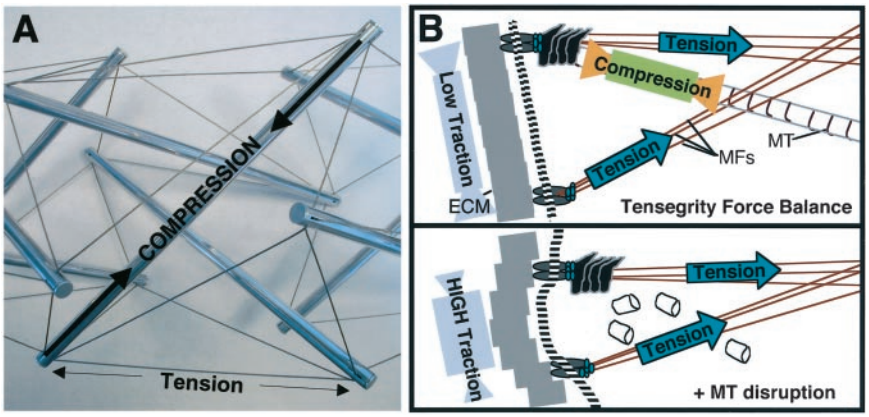

Figure 4. A, Tensegrity sculpture by the artist, Kenneth Snelson. This structure is composed of a series of isolated steel struts that are interconnected with a continuous series of metal cables. Struts bear compression that is generated by the surrounding network of tensed cables. Mechanical stability of this structure, like that of the cytoskeleton in a living cell, depends on the level of preexisting tension (prestress) or "tone" in the network. B, Schematic diagram of the complementary, tensegrity force balance between tensed microfilaments (MFs), compressed microtubules (MTs), and the ECM in a region of a cellular CSK array (for simplicity, the tensed intermediate filaments are not shown). Compressive forces borne by the microtubule struts are transferred to ECM adhesions when microtubules are disrupted (bottom), thereby increasing substrate traction, as observed in experiments with living cells (see text).

represents a developmental switching mechanism that may play a fundamental role during both control of angiogenesis and development of larger blood vessels.

Mechanochemical Transduction

Cellular Tensegrity Model

If mechanical distortion of cell shape can alter cellular behavior, then how might this work at the molecular level? It has been difficult to see how physical forces can impact on signal transduction and gene expression because most investigators still envision the cell effectively as an elastic membrane surrounding a viscous or elastic cytoplasm. Thus, for many years, the mechanism of mechanotransduction remained a black box. In contrast, we proposed a model for mechanoregulation that was based on the idea that cells were not balloons filled with "molasses or jello," and instead, that they responded mechanically as discrete filamentous networks.[2,22–25] This idea was based on the discovery in the 1970s that cells appear to be stabilized from within by a continuous cytoskeletal network composed of three classes of molecular filaments: contractile microfilaments, microtubules, and intermediate filaments.

In addition, we proposed that living cells structure their CSK using a particular form of architecture that comes from the Buckminster Fuller world of geodesic architecture and is known as tensegrity (shortened from "tensional integrity").[2,22,23] These structures gain their stability from continuous tension, rather than from continuous compression as in man-made, brick-on-brick constructions. In tensegrity structures, inward-directed tensional forces that are distributed to all elements are resisted by a subset of elements that cannot be compressed. In this manner, a dynamic force balance is established and an internal preexisting tensile stress (prestress) is generated that stabilizes the entire structure. The simplest examples of tensegrity are the sculptures of Kenneth Snelson, which are composed of multiple compression-resistant struts (steel bars) that are apparently suspended in air without touching; in reality, they are interconnected, tensed, and stabilized in space through interconnection with a continuous series of high tension cables (Figure 4A). Another more familiar example is the human body, which gains its shape stability by interconnecting multiple compression-resistant bones with a continuous series of tensed muscles, tendons, and ligaments.

In the cellular tensegrity model (Figure 4B), the shape of the cell is similarly stabilized through a mechanical force balance in which microtubule struts and ECM adhesive tethers resist the pull of contractile microfilaments.[22,23] In this model, intermediate filaments that interconnect at many points along microtubules, microfilaments, and the nuclear surface act as molecular guide wires and suspensory cables to tensionally stiffen the whole CSK and nuclear lattice. The simplest way to envision this model is to think of a camp tent. To stabilize the form of the tent and provide it with "shape stability" (ie, stiffness), we push the tent membrane up using tent poles and then further tense the membrane by placing adhesive anchors (tent pegs) into the ground. If there is a tree branch above the top of the tent pole, we also can place another tether to this overlying support; once this is in place, the tent pole could be removed without significant loss of shape stability. Thus, in this tent analogy, the compression-resistant poles and external tethers act in a complementary manner to resist the tension in the tent membrane that connects all the structural elements. It is through this dynamic balance of forces that the tensional prestress is generated that stabilizes the tent's form.

If the CSK is organized like this tent, then if you were to break the microtubule struts (tent poles), the force they normally carried would be transferred onto the cell's external adhesive tethers and the tree branch would be wrenched downward. In contrast, if all the filaments of the CSK experience tension (eg, like a bunch of tensed rubber-bands), then if you were to cut any of the filaments, tension on the adhesive substrate would rapidly dissipate and the tree branch might leap back up to its original resting position. In fact, many studies have shown that when microfilaments or intermediate filaments—the tension elements in the model—are chemically disrupted, cell tractional forces exerted on ECM anchors decrease.[26–28] On the other hand, when microtubules—the struts in the model—are disrupted, traction on the ECM substrate rapidly increases in many cell types.[29–31]

These results directly support the tensegrity model (Figure 4B). However, one caveat is that microtubule disruption also has been shown to activate myosin light chain kinase.[30] Thus, some interpret this to mean that the observed increase in ECM traction is controlled chemically (eg, via tubulin monomer release) and not mechanically via a tensegrity force balance.[30] In recent studies, we have been able to unequivocally demonstrate that microtubule disruption results in an increase in tractional forces exerted on the substrate even under conditions in which myosin light chain phosphorylation does not change.[31] This finding, in combination with microscopic visualization of green fluorescent protein (GFP)–labeled microtubules that demonstrates end-on compressive buckling of microtubules in living cells,[31,32] has confirmed that mammalian cells do indeed use tensegrity architecture to stabilize their form.

Importantly, working with Dimitrije Stamenovic (Department of Biomedical Engineering, Boston University, Boston, Mass), we have developed a mathematical basis to explain this fundamental mechanical behavior starting from first principles.[33,34] This theoretical model has been progressively revised and strengthened by the Stamenovic group to include semiflexible microtubule struts linked to tensed microfilament cables[35,36]; one embodiment of the model also includes tensed intermediate filament linking cables.[28] This physics-based tensegrity model has led to multiple a priori predictions relating to both the static and dynamic mechanical behavior of living cells that have been confirmed in experiments with various types of mammalian cells.[26,27,36–42] Thus, the tensegrity model appears to be the most unified model of cell mechanics available at the present time.

Integrins as Mechanoreceptors

The importance of the tensegrity model for mechanoregulation is that it suggested that adhesive receptors that physically link extracellular support scaffolds (eg, ECM) to the internal CSK may function as mechanoreceptors.[2,24,25] In other words, adhesion receptors, such as integrins, should provide preferred paths for mechanical signal transfer across the cell surface, whereas other transmembrane receptors that do not link to the internal CSK should dissipate stress locally and thus fail to transmit the same signals.

To test this hypothesis, we developed a magnetic twisting device in which living cells were allowed to bind to ferromagnetic microbeads (1 to 10 μm diameter) that were precoated with specific receptor ligands (eg, synthetic RGD-peptides, anti-receptor antibodies, growth factors).[37–39] A few minutes after binding to the cell surface, and while the beads were still on the outside of the cell, magnetic twisting forces (torque) were applied to the beads while simultaneously measuring induced bead rotation. This approach allowed us to apply controlled shear stresses directly to one class of receptors, independently of other receptors on the same cell surface. More recently, we developed a magnetic tweezer that allows us to apply linear tensional stresses in a similar manner.[43]

Both magnetic manipulation techniques revealed the same result: integrins and other adhesion receptors that link to the internal CSK (eg, PECAM, E-selectin, cadherins) provide a greater degree of mechanical coupling across the cell surface relative to other transmembrane receptors. Work we performed in collaboration with Dr Ning Wang (Harvard School of Public Health, Boston, Mass) has shown that these receptors differed in their ability to mediate transmembrane mechanical coupling in the following manner: integrin β_1>PECAM>integrin $\alpha_v\beta_3$>E-selectin>integrins α_5, α_2, α_v>>>acetylated-low density lipoprotein receptor>HLA antigen. These differences in the mechanical coupling capacity appear to depend directly on the ability of the integrin to organize a focal adhesion linkage to the internal CSK. For example, genetic disruption of the focal adhesion protein, vinculin, results in a large drop in mechanical coupling independent of integrin binding, and this loss can be restored by transfecting the cells with intact vinculin protein.[16,43] Similarly, when cells are mechanically stressed through nonactivating integrin antibodies, mechanical coupling is weak, whereas when soluble RGD peptide is added to the same cells to activate these ligated integrin receptors and induce focal adhesion formation, the mechanical linkage rapidly rigidifies and transmembrane mechanical signaling proceeds.[44] Thus, the differences in mechanical coupling by distinct integrins we observed are likely not due to simple variations in integrin expression levels.

Molecular Basis of Mechanochemical Transduction

The finding that cells use tensegrity to stabilize the form of their CSK, and that adhesion receptors mechanically couple the CSK to ECM and neighboring cells suggested a potential mechanism for mechanochemical transduction.[23,24] In this type of system, physical distortion of the tissue or ECM would change force distributions across adhesion receptors and result in both local and global restructuring of the tensionally integrated CSK network. Similar changes may be produced from within by altering CSK tension while maintaining the resisting anchors constant: both would produce CSK remodeling throughout the cell and hence, provide a potential mechanism for signal integration in both time and space.

In fact, we were able to confirm that application of distending forces to cell surface integrins results in transmembrane force transfer, coordinated realignment of actin microfilaments within the cytoplasm, and recruitment of protein synthetic complexes to sites of force application in living cells.[41,45] Even more interesting was that similar application of force also resulted in movement of mitochondria that attach to microtubules deep within the cytoplasm.[31] Application of greater distending forces to larger ECM adhesions even induced molecular realignment (as visualized by birefringence microscopy) within nucleoli inside nuclei of living cells.[41] Thus, cells do behave like discrete filamentous networks in terms of how they transmit mechanical forces through their cytoplasm and nucleus, and not like a bulk gel or fluid.

Solid-State Biochemistry on the Cytoskeleton

Importantly, the CSK does more than provide shape stability to the cell; it also orients much of the cell's metabolic machinery. Many of the enzymes and substrates that mediate

DNA synthesis, RNA processing, protein synthesis, glycolysis, and signal transduction function when physically immobilized on insoluble molecular scaffolds within the cytoplasm and nucleus.[46] This form of solid-state biochemistry helps to explain the incredible efficiency of biochemical reactions observed in living cells because it is not diffusion-limited. Moreover, it provides a mechanism to compartmentalize potentially contradictory biochemical functions within the same cell. It also has extremely important implications for how mechanical signals may be transduced into a biochemical response.

If cells use tensegrity architecture and a distending force is quickly applied to cell surface receptors, then the load-bearing filaments of the CSK will either distort or break. Our experiments suggest that the filaments do not break and that mechanical continuity is maintained when forces are applied to integrins that form strong focal adhesion linkages to the CSK, whereas breakage can be observed when the same stress is applied to transmembrane metabolic receptors.[31] If the CSK filaments and associated load-bearing molecules that connect to integrins distort, then either some or all of the molecules that comprise these structures must similarly change shape. If one changes molecular shape, then the biophysical properties of the molecule will be altered. For example, past theoretical work predicted that decompressing a microtubule will change a key thermodynamic parameter—the critical concentration of tubulin—and thereby promote microtubule polymerization.[47] However, it was never clear how a microtubule could bear compression in a living cell. Our work on cellular tensegrity has revealed that ECM tethers and microtubule struts function in a complementary manner to resist CSK tension. If this is true, then when distending forces are applied to integrins, the microtubules would be decompressed and microtubule polymerization should be promoted. In fact, exactly this type of behavior has been demonstrated in both nerve cells[48,49] and smooth muscle cells[50] in culture.

Altering molecular shape via distension of integrin linkages to the CSK also may alter kinetic parameters. For example, imagine a spring fixed at this base that vibrates at a certain frequency. If the size, shape, or center of gravity of this spring were to be altered, its vibration frequency would change accordingly much like a metronome. In an analogous manner, altering molecular shape will change the kinetic behavior of the molecule and thus alter its biochemical properties, including enzyme reaction rates. In fact, stress-sensitive ion channels have been identified that alter their kinetics when mechanically stressed. Interestingly, even these membrane components appear to experience mechanical forces through transmission via adhesion receptors and associated CSK elements (see review[51]).

Mechanochemical Transduction via Integrins

To explore this potential mechanism for mechanochemical transduction in greater detail, we focused on the role of integrin receptors. There were three reasons for this approach: (1) integrins provide a preferred path for transmembrane mechanical force transfer, (2) integrins physically couple to the CSK via formation of a focal adhesion complex, and (3) we and others previously showed that the CSK backbone of the focal adhesion serves to orient much of the cell's signal transduction machinery, including tyrosine kinases, inositol lipid kinases, ion channels, and certain growth factor receptors.[52–54]

We therefore asked whether application of controlled mechanical stresses directly to surface integrin receptors using the magnetic twisting device could result in stress-dependent activation of intracellular signaling in endothelial cells. We focused on the cAMP signaling system because this was the first transduction pathway shown to be mechanosensitive.[55] These studies revealed stress-dependent activation of adenylyl cyclase resulting in increases in intracellular cAMP, nuclear translocation of the catalytic subunit of protein kinase A, phosphorylation of the transcription factor, CREB, and associated induction of transcription of a reporter gene driven by multiple cAMP response elements.[44] In contrast, none of these signaling responses were observed when the same stress was applied to transmembrane metabolic receptors that failed to form focal adhesions in the same cells. Thus, integrins appear to mediate mechanochemical transduction by preferentially transferring mechanical stress to signaling molecules that are immobilized on the CSK framework of the focal adhesion. In this manner, the focal adhesion represents a point of convergence for the three different classes of signals that regulate cell behavior: soluble cytokines, insoluble adhesion molecules, and mechanical forces.

Various laboratories have confirmed that integrins play a central role in mechanotransduction and cardiovascular development in studies with cultured vascular endothelial cells, smooth muscle cells, and cardiac myocytes as well as whole tissue and organ experiments in living animals. Signaling activities that have been shown to be modulated by mechanical distortion or fluid shear stress and to be mediated by integrins or focal adhesions in these cells include Src, FAK, ERK1/2, Shc, Grb2, PKC, NF-κB, Akt, PI-3 kinase, PI-5 kinase, calcium, cAMP, various stress-sensitive ion channels, actin polymerization, and expression of genes encoding PDGF, endothelin-1, and sterol regulatory element-binding protein-1.[44,51,56–68] Genetic disruption of integrin function in cardiac myocytes also has been shown to lead to impaired contractility and loss of normal heart function in transgenic mice.[69]

Whole Cell as Mechanosensor

Although most investigators in the mechanotransduction field strive to identify the molecules that mediate mechanochemical conversion, the reality from a physiological perspective is that the whole cell is the mechanosensor. For example, twisting integrins on the surface of round cells on small islands produces the same increase in cAMP as in spread cells on large adhesive islands. Yet, the round cell integrates this input with others signals and switches on an apoptotic program while spread cells grow (Figure 2). Thus, to understand how mechanical forces exert local developmental control, we need to further dissect the mechanism of distortion-dependent regulation of cell behavior.

Study of the process of ECM-dependent growth control in capillary endothelium has revealed the existence of a discrete

shape-dependent restriction point at the G1/S border of the cell cycle.[11,12] Cells that are restricted from spreading fail to downregulate the cdk inhibitor, p27, and upregulate the activator, cyclin D1, even though they are stimulated with optimal concentrations of both soluble growth factors and insoluble ECM. A similar block in these key cell cycle regulators results when the actin cytoskeleton is disrupted using cytochalasin D before the G1/S transition, but not when the same drug is added a few hours later (still before S-phase entry). Under these conditions, the cells enter S phase normally even though the entire actin CSK is disrupted and cell rounding is induced. Thus, the shape-dependent cell cycle restriction point also appears to be an actin CSK-dependent control point and similar control points have been identified in other cell types.[70,71] Interestingly, the same cell cycle block was obtained by treating cells with butanedione 2-monoxime.[11] This drug does not disrupt CSK filaments or change cell shape, instead it inhibits tension generation and thus decreases tensional prestress within the CSK. Disruption of intermediate filaments, which similarly decreases tensional prestress and CSK stiffness without changing cell shape, also inhibits cell proliferation.[28]

Additional studies have confirmed that CSK microfilaments and microtubules also contribute actively to apoptotic control in capillary cells via modulation of Akt and bcl2 signaling[14] and that CSK tension generation plays an important role in determining the direction of lamellipodia extension.[15] This latter result is consistent with the finding that cells that lack the focal adhesion protein, vinculin, which normally mediates mechanical coupling between integrins and the actin CSK, fail to extend lamellipodia even when injected with constitutively active *rac*.[16,72] Thus, tension transfer between the CSK and ECM across integrins may act locally to control the ability of this small G protein to manifest its lamellipodia-inducing activity.

Cell and Developmental Control Lies in the Balance of Forces

Taken together, these findings suggest that complex cellular behaviors within developing tissues, such as growing vascular networks, are controlled through local changes in the cellular force balance.[23,73] Physical distortion of tissues or their ECM will result in CSK remodeling and alter intracellular biochemistry via local changes in thermodynamic and kinetic parameters. The inverse is also true: changes in CSK tension will result in remodeling of structural scaffolds within the ECM and within neighboring cells, thereby altering biochemistry outside the cell. Examples include demonstration that fibronectin fibril assembly is tension-dependent[74] as well as the finding that cell distortion impacts on the production of multiple ECM proteins.[20,75] However, if we go back to our initial hypothesis, then the most interesting implication is that local changes in the cellular force balance may mediate pattern formation during tissue morphogenesis.

In the case of angiogenesis (Figure 1), capillary sprout formation can be initiated by addition of agents, such as angiogenic factors[4] or ECM modulators,[76] that locally accelerate ECM turnover. Endothelium, adjacent pericytes, and underlying fibroblasts cells all exert contractile forces on their ECM adhesions, which must come into balance to ensure tissue stability. Thus, the basement membrane is a pretensed or prestressed structure. Because stromal cells generally exert higher levels of tension, partial enzymatic degradation of a small region of the ECM that significantly compromises its structural integrity (stiffness) would result in physical extension and thinning of the basement membrane, as observed in regions of capillary initiation. Endothelial cells adherent to this strained microdomain of the basement membrane would experience an increase in tension exerted on their ECM receptors—the integrins. These forces would be transmitted across the cell surface and to CSK filaments that physically couple to integrins within specialized focal adhesion complexes. Based on work from our laboratory and others, increasing tension on integrins will both induce CSK restructuring and activate various signal transduction pathways through tension-driven remodeling of these focal adhesion connections. These mechanical signals will then modulate ongoing signaling cascades that are elicited by soluble cytokines and ECM-induced clustering of integrin receptors within the same focal adhesions.

In this manner, capillary endothelial cells may modulate their response to soluble angiogenic factors by sensing ECM and mechanical cues in their local microenvironment. The cells directly adjacent to the region of basement membrane thinning that experience increased tension on their focal adhesions will be able to respond by extending new migratory processes and sprouting in the direction of the pull. As cell spreading is promoted, these cells also will be able to progress through the cell cycle and proliferate, even though they remain part of a continuous endothelial monolayer. In contrast, neighboring cells only microns away in the same monolayer that remain adherent to intact basement membrane will feel no change in CSK tension or associated distortion and, hence, will remain quiescent. In cases where newly formed capillaries later regress, this may result from a net increase in basement membrane degradation, which further compromises scaffold integrity to the point where cell retraction and apoptosis are induced, even though the microenvironment is saturated with soluble angiogenic mitogens. Thus, in this model of tissue patterning, branch formation is not solely due to local release of a growth factor or localized expression of a particular type of ECM component; rather, it is driven by a differential in cell and tissue mechanics acting in an environment that contains these chemical cues.

To begin to explore the possibility that this form of morphogenetic control is utilized during embryogenesis, we recently performed studies using a mouse lung rudiment model.[77] When lung rudiments from embryonic day 12 were placed in culture, they underwent normal epithelial branching over the subsequent 48 hours. When these rudiments were treated with the Rho kinase inhibitor, Y27632, to decrease CSK tension generation, morphogenetic branching was suppressed. Even more interestingly, when 12-day rudiments were treated with the rho activator, CNF-1, to increase CSK tension, branch formation increased; both more branches and deeper clefts were observed. Recent biochemical studies indicate that this effect on morphogenesis correlates more closely with effects on myosin light chain phosphorylation

(ie, tension generation) than on *rho* activity per se. Thus, these findings provide the first evidence to suggest that changes in the CSK force balance may have a direct effect on developmental patterning in vivo. The possibility that a similar mechanochemical control mechanism functions during angiogenesis is supported by the findings that capillary tube formation in vitro is influenced by local changes in ECM mechanics or adhesivity[8,78]; that capillary sprout elongation can be promoted by local application of tensional stresses in vitro[79]; and that endothelial cell death and capillary regression can be induced in a growing capillary bed using chemical agents that induce complete basement membrane breakdown and associated endothelial cell rounding in vivo.[6,7]

Relevance for Vascular Development and Cardiovascular Physiology

Our exploration of potential mechanical control mechanisms during capillary development has led to the identification of fundamental design principles that guide how mammalian cells and tissues structure themselves. Experimental confirmation that cells utilize tensegrity architecture to stabilize their shape and remodel their CSK framework provides a new handle on cell mechanics for engineers and physicists as well as molecular cell biologists. Computational models now exist that can predict complex mechanical behaviors in living cells; these models may lead to new predictions and hence, assist in the design of new experiments. Of even greater importance is the insight that the tensegrity model has provided into the molecular and biophysical basis of mechanotransduction. For more than a century, scientists have known that mechanical forces sculpt tissue form, yet there was little understanding of the underlying mechanism. The studies described here helped to identify integrin receptors and their links to the CSK as key players in this process, a discovery that has now been confirmed in various cell types and species.[24,25,51,56–69,80,81]

From the perspective of vascular biology, our work suggests that ECM, integrins, CSK molecules, and the tension generation machinery represent potential targets for therapeutic manipulation of vascular development. The finding that integrin antagonists are potent angiogenesis inhibitors[82] and that modulators of CSK microtubules can inhibit angiogenesis[83] as well as vascular smooth muscle growth and motility[84] add support to the physiological importance of this work. The reports that other angiogenesis inhibitors bind to elements of the contractile machinery[85–87] are especially intriguing in this context. The novel point here is that modulation of CSK structure and function may have potent effects on behaviors other than motility and contractility, including growth, differentiation, apoptosis, and pattern formation.

Rules similar to those we uncovered in our studies on control of angiogenesis may also guide restructuring of larger blood vessels and of the heart in response to changes in mechanical parameters, ECM remodeling, or CSK alterations in cardiovascular diseases, such as hypertension, atherosclerosis, or various cardiac myopathies. For instance, although atherosclerotic plaques are known to form preferentially in regions of disturbed fluid shear stress at vascular bifurcations, the mechanism for this response remains unknown. Many studies demonstrate that individual endothelial cells can sense alterations in fluid shear and respond by altering signal transduction and gene expression.[80,81] However, most investigators seek to find *the* mechanoreceptor molecule on the apical cell surface. Our work on cellular tensegrity, combined with the finding that intermediate filaments transmit apical fluid shear deep into the CSK,[88] suggest an alternative mechanism whereby apical stresses result in subsequent distortion of the cell's basal integrin adhesions that, in turn, would mediate mechanical signaling. Moreover, the tensegrity model also provides a mechanism to explain why altering the prestress in the actin cytoskeleton can modulate shear stress-dependent activation of genes, such as endothelin-1.[61] Once again, the novel point here is that the whole cell is the mechanosensor and that cellular control results from physical interplay between various molecular components.

Insights from the tensegrity model, which suggest that the mechanical and contractile behaviors of cells are controlled through collective interactions between microfilaments, microtubules, intermediate filaments, and the cell's ECM adhesions, also may have direct relevance for cardiovascular physiology. For example, in both animal models and human patients with severe right and left ventricular pressure overload hypertrophy, increased microtubule density appears to cause cardiac muscle cell dysfunction through increased viscous loading of active myofilaments, and this contractile dysfunction is normalized by microtubule depolymerization.[89,90] Mechanical measurements of isolated cardiac cells confirmed that microtubule depolymerization using colchicine returned both the stiffness and apparent viscosity of pressure overload-hypertrophied right ventricular cells to normal levels, whereas microtubule hyperpolymerization using taxol increased the stiffness and apparent viscosity of normal cells.[91]

Importantly, as suggested by the tensegrity model, intermediate filaments also have been found to play an important role in control of cardiac and smooth muscle cell contractility. Genetic disruption of the intermediate filament protein, desmin, in mice results in a decrease in active force generation within cardiac muscle cells and associated cardiac myopathy.[92] Analysis of vascular function in similar mice revealed that pressure-induced (myogenic) tone was unchanged, but agonist-induced tone decreased in resistance arteries.[93] Moreover, shear stress (flow)– and acetylcholine-induced, endothelium-dependent dilation, as well as endothelium-independent dilation, were also decreased in these same vessels. Intermediate filaments also contribute to control of cell contractility[27] and the endothelial cell response to fluid shear stress.[88]

Thus, although the paradigm in the contractility field has been that cell contraction is a direct result of actomyosin interactions, these studies clearly demonstrate that intermediate filaments and microtubules also contribute to the efficient control of cardiac contractility, vascular tone, and endothelial shape stability. Similarly, although accumulation of ECM has been viewed as an end result of the hypertensive process, more recent work shows that integrins contribute significantly to control of vasoconstriction in vivo[68]; that changes in cell-ECM binding and mechanical force transfer

across integrins modulate vascular smooth muscle cell contractility in vitro (T.R. Polte and D.E. Ingber, unpublished observations, 2002); and that the ECM plays an active role in the maintenance of normal cardiac function.[94] In fact, the tensegrity model also may have relevance at higher (tissue/organ) levels of organization within the heart. For example, establishment of a force balance between the contractile myocardium and the resisting elastin bundles within the ECM of the heart organ is responsible for integrating motion throughout the atria and ventricles and for ensuring for the elastic recoil that is so key to cardiac function. Thus, maintenance of an optimal blood supply apparently depends on complex mechanical interplay between various molecular networks that provide mechanical support to the cells and tissues that comprise the vasculature.

Implications for the Future

These new perspectives clarify that complex cell and tissue behaviors result from collective interactions among many different molecular components. For this reason, existing reductionist descriptions that focus on single molecules or individual signaling pathways will not prove sufficient as we try to attempt to model complex biological systems as a whole and to understand living tissue physiology. In contrast, the tensegrity model, which incorporates this concept of collective behavior, may provide a mechanism to place these new insights into a more physiological perspective. It also may assist in the design of future experiments to tackle this problem at both the biophysical and molecular levels.

Confirmation of local mechanical control of tissue patterning awaits development of new methods to attack this problem using in vivo model systems. These techniques will include techniques for applying controlled stresses directly to specific molecules on individual cells within distinct tissue locations as well as noninvasive, real-time readouts of critical cellular responses. However, our early results in the lung and angiogenesis models provide strong evidence in support of the concept that local changes in tissue structure and mechanics contribute significantly to the developmental response. This then leads us to the ultimate question: how can a signal as nonspecific as cell distortion produce the same cell fates or phenotypes (eg, growth, differentiation, apoptosis) as a specific stimulus, such as a growth factor that binds to high-affinity receptors with angstrom resolution?

To address this question, we must confront various challenges in the near term. First, we must identify the key molecular elements that contribute to the cellular response to stress in different cell types and tissues. Second, we need to understand how these elements link to specific mechanotransducing molecules. Finally, we will have to decipher how the cell integrates these signals with other cues elicited by chemical regulatory factors. In the process, we will hopefully identify novel molecules that selectively mediate cellular mechanosensation and mechanochemical transduction and, in the process, uncover potentially novel targets for therapeutic intervention in a wide variety of vascular diseases.

However, to fully address this question, we will need to go beyond current concepts of linear signaling pathways: reductionism will have to be put aside. We will need to pull in paradigms and approaches from other fields, such as physics and mathematics, which already have mechanisms to deal with the fundamental question of how complex behaviors emerge from collective interactions. We will need to analyze the function of dynamic networks, deal with questions relating to control theory, understand hierarchical contributions to behavioral control and, in most general terms, begin to understand how structural networks impact on information processing networks within living cells. The tensegrity paradigm combined with recent insights from complex systems science that suggest that cell phenotypes are analogous to mathematical attractors[95] may provide a handle with which to approach these complex issues. But complete understanding of the machinery of vascular development will undoubtedly require combination of all of these techniques and an equal merging of computational approaches with experimental methods so that we may directly discern the function of networks of interacting molecules within living vascular tissues.

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