FINAL REPORT

TITLE:	Mechanotransduction through Integrins
GRANT #:	NAG 2 -1501
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SUMMARY OF RESEARCH

The goal of this project was to characterize the molecular mechanism by which cells recognize and respond to physical forces in their local environment. The project was based on the working hypothesis that cells sense mechanical stresses through cell surface integrin receptors and through their interconnections with the underlying cytoskeleton. Work completed and published in past funding period had provided direct support for this hypothesis. In particular, we demonstrated that application of mechanical stresses to activated integrin receptors (but not inactive integrins or other control transmembrane receptors) resulted in stress-dependent activation of the cAMP signaling pathway leading to gene transcription. We also showed that this form of mechanotransduction requires activation of heterotrimeric G proteins. In this grant, our specific aims included: 1) to characterize the signal processing capabilities of different integrins and other cell surface receptors, 2) to identify heterotrimeric G proteins that mediate cAMP signaling by stresses applied to integrins, 3) to identify molecules that mediate transmembrane mechanochemical coupling between integrins and G proteins, and 4) to use genome-wide gene expression profiling techniques to identify other genes and signaling pathways that are activated by mechanical forces transmitted over specific cell surface receptors. Elucidation of the mechanism by which cells sense mechanical stresses through integrins and translate them into a biochemical response should help us to understand the molecular basis of the cellular response to gravity as well as many other forms of mechanosensation and tissue regulation.

Accomplishments:

To determine whether mechanical signals are transferred across the cell surface over discrete molecular pathways, we developed a magnetic twisting cytometry (MTC) technique in a past grant period. This method can be used to apply controlled mechanical

stresses directly to specific cell surface receptors without producing global changes in cell shape or altering fluid flow. With this device, torque is applied to membrane-bound ferromagnetic microbeads (4.5 μm in diameter) that are coated with ligands or antibodies for different cell surface receptors by first magnetizing the beads in one direction and then applying a weaker twisting magnetic field that does not remagnetize the beads in the perpendicular orientation. The cellular deformation that results in response to application of this shear stress (twisting of the bead and tangential shearing of the adherent cell surface receptors) is determined by simultaneously quantitating bead rotation (angular strain) using an in-line magnetometer. Changes in biochemical signal transduction and gene expression induced by these stress also may be measured in parallel.

We used MTC to demonstrate that integrins preferentially transfer mechanical stresses across the membrane and to the cytoskeleton relative to other transmembrane receptors, including metabolic acetylated-low density lipoprotein (AcLDL) receptors, HLA antigens and FGF receptors. We also confirmed that different integrins (β1, β3, αV, α5, α2) and cell-cell adhesion molecules (e.g., E-selectin, PECAM, cadherin) that also link to the internal cytoskeleton similarly mediate force transfer across the cell surface, although the efficiency of coupling varied considerably from receptor to receptor. In addition, we demonstrated that similar mechanical coupling between integrins and cytoskeleton is observed in many cell types and that mechanical responsiveness does not require plasma membrane continuity. Taken together, these results confirmed our hypothesis that integrins act as mechanoreceptors that may be important for gravity sensation. The challenge now was then to understand how cells respond to mechanical signals transmitted over these receptors.

Cells mechanically adapt to externally-applied forces by producing a stress-induced strengthening response: the mechanical stiffness of the focal adhesion (FA) increases in parallel with the applied stress. This adaptation to mechanical stress is important because it minimizes cell injury (e.g., prevents membrane tearing), ensures cell viability, and plays a critical role in cell movement. Thus, understanding the molecular basis of this response is important for the field of cellular mechanotransduction. Importantly, in past grant periods, we showed that cytoskeletal stiffening results when mechanical forces are applied to cell surface integrins, but not other transmembrane receptors (e.g., metabolic receptors, growth factor receptors, HLA antigen) using MTC or ligand-coated pipettes or beads moved with a micromanipulator. In this grant period, we showed that these adaptive, stress-dependent increases in cell stiffness mediated by integrins are partly due to associated changes in FA assembly. For example, binding of magnetic beads to b1 integrins induces recruitment of integrins and associated FA proteins, including vinculin, talin, α-actinin, and F-actin, to the site of bead binding. We also showed that cells from mice lacking vinculin exhibited a large drop in transmembrane mechanical coupling that is independent of integrin binding as measured using MTC or an "electromagnetic tweezer" that we developed which applies tension rather than shear to receptor-bound magnetic microbeads. Most importantly, the stiffness of the FA was fully restored when intact vinculin protein was transfected back into the cells, whereas vinculin fragments that failed to bind both integrins and other FA proteins were only partially effective.

Although some of the cell strengthening response is clearly due to activation of signal transduction and resulting increases in FA assembly, part of this response also may be due to passive material properties of the FA (and associated cytoskeleton) once it has formed. For example, prestressed tensegrity models composed of sticks and strings that mimic many properties of the tensed cytoskeleton display similar stiffening behavior, as we showed in past grant periods. In fact, we showed that cell stiffening induced by stress application to integrins using MTC can be altered by chemically modulating cytoskeletal prestress, or by disrupting microtubules and intermediate filaments, as well as microfilaments that directly connect to integrins. We also showed that certain cell stiffening responses proceed normally at 4°C. Thus, the cell's adaptive response to applied mechanical loads may involve multiple mechanisms, including both changes in passive material properties of the prestressed cytoskeleton and active molecular remodeling events driven by mechanochemical signal transduction.

In the past grant period, we developed yet another micromagnetic technique – a "magnetic microneedle" to examine how individual FAs within single cultured cells mechanically adapt to both static and dynamic force regimens, and how the state of integrin activation, FA assembly, cytoskeletal prestress and the passive material properties of the cell contribute to these responses. The microneedle is composed of a stainless steel needle fastened to a permanent magnet; the needle tip is dynamically positioned using a computer-controlled micromanipulator on a microscope stage. To analyze the mechanical response of cells to force, adherent endothelial cells were allowed to bind magnetic microbeads (4.5 μm diameter; either superparamagnetic or ferromagnetic) that were pre-coated with specific receptor ligands (e.g., synthetic RGD-peptide for integrins) for 10 min prior to force application. By using automated computer control to rapidly move the needle away (600 mm) from the cell and then to return to the same position, tensional forces (0 to 250 pN) could be applied for as brief as 1 second, both as single pulses or repeated pulse bursts. Resulting bead displacements are determined with nanometer resolution using real-time optical microscopy in conjunction with computerized image analysis.

Our microneedle experiments revealed that cells reinforced their structural linkages to the RGD-coated magnetic beads through integrins and stiffened in response to a force (130 pN) pulse of 3 sec duration. Comparison of individual beads revealed that beads that failed to recruit FA molecules, such as GFP-labeled actin, vinculin or paxillin, exhibited a much smaller strengthening response and displaced more. When similar force was applied through non-activated integrins using K20 anti-β1 integrin antibodies that bind but do not ligate the receptors or recruit GFP-labeled FA proteins, the strengthening response was not observed, however, stiffening could be restored by adding soluble RGD peptide to ligate and activate the K20-bound receptors. Moreover, when exposed to a train of multiple similar force pulses (130 pN; 3 sec on/ 4 sec off), cells further stiffened their adhesions by approximately 20% ($p<0.05$); this response saturated by the third pulse and reversed within 5 min after removal of stress. Importantly, both of these early responses to stress applied through activated integrins were suppressed by first dissipating pre-existing tension (prestress) in the cytoskeleton using inhibitors of the rho-

associated kinase, ROCK (20 mM Y27632), or of myosin ATPase (10 mM; 2,3-butanedione 2-monoxime; BDM). Microscopic analysis of large-scale bead movements (another property that is important for cell translocation and motility) also revealed that RGD-beads exposed to prolonged forces (>15 sec) first moved toward the magnet, and then they stalled and reversed their direction. This ability of cells to pull beads against the magnetic force gradient was prevented when beads were coated with control ligands, such as K20 or AcLDL that fail to form integrin-cytoskeletal linkages, or when cytoskeletal tension was inhibited using Y27632 or BDM.

Together, these data indicate that individual FAs exhibit distinct, active strengthening responses depending on the dynamic nature of the mechanical stimulus, that individual FAs on the same cell can exhibit different responses to stress, and that integrin activation, FA assembly and cytoskeletal prestress are all important regulators of these responses. Moreover, rho-dependent signaling through ROCK mediates these responses. Importantly, we also have generated various genetic probes to manipulate the rho pathway and have successfully used these constructs, as well as pharmacological modifiers, in cultured endothelial cells. For example, we can selectively inhibit or stimulate stress fiber formation and FA assembly by delivering C3 exoenzyme or constituitively active rho(V14) (both at 5 mg/ml) into cells using a protein transfection technique (BioPORTER reagent; Gene Therapy Systems). We also standardly use drugs and similar gene constructs in our laboratory to modulate ROCK, mDia1, and rac1, and we established a rhotekin assay in the lab for quantitating rho activity directly.

Because further analysis of cell responses to dynamic force regimens would be helpful for this field, we recently developed an improved modification of this technique that uses a "micromachined electromagnet". The solenoidal current powering the electromagnet is supplied from a variable gain amplifier that is controlled through a Macintosh data acquisition system programmed using LabVIEW (National Instruments). Applied force frequencies can range from 0 to 1000 Hz and utilize virtually any desired wave form (e.g., sinusoidal versus square wave). Importantly, the MTC device which applies shear, rather than tension, also has been modified so that we can provide oscillatory forces over a similar frequency range.

Cells also change various biochemical signaling activities, including gene expression, when mechanically stressed, however, less is known about this mechanism than the cell strengthening response. We and others have shown that multiple signaling molecules that are activated by integrins, as well as growth factors, are recruited to the FA within minutes after integrin clustering is induced. Moreover, isolated cytoskeletal fractions that are enriched for FAs retain many of these signaling activities even after removal of most membrane lipids. Thus, the cytoskeleton framework of the FA appears to represent a major site for signal integration between growth factor and ECM-based signaling pathways. In addition, as described above, integrins also transmit stresses across the cell surface, and hence they may focus this mechanical energy on FA-associated signaling molecules.

Thus, our 2nd working hypothesis has been that the FA represents a potential site for converting mechanical signals into biochemical responses. Importantly, we were able to confirm this hypothesis in our past NASA-funded studies. For example, we used high resolution *in situ* hybridization to show that mRNA and ribosomes are rapidly recruited to FAs in a stress-dependent manner when forces are applied to integrins, but not other transmembrane receptors using MTC. Different integrins (e.g., $\alpha5\beta1$ vs $\alpha V\beta3$) also differed in their ability to support this response. In addition, we showed that stress application to integrins using MTC (15.6 dyn/cm^2 or ~300 pN torque/bead) also elicits a cAMP signaling response leading to gene activation. Specifically, twisting activated $\beta1$ integrin receptors resulted in stress-dependent increases in cyclic AMP, nuclear translocation of the catalytic subunit of protein kinase A, phosphorylation of the transcriptional regulator CREB, and transcription of a gene reporter driven by the cAMP response element in endothelial cells and fibroblasts. In contrast, twisting control transmembrane AcLDL receptors or inactive $\beta1$ integrins (using K20 antibodies) failed to produce these signaling events. Furthermore, mechanical signaling could be restored in cells using beads coated with the K20 antibodies by simultaneously adding a soluble RGD ligand that ligates and thereby, activates the twisted integrin receptors. Taken together, these results clearly demonstrated that generalized membrane distortion alone is not sufficient to activate this mechanotransduction response, whereas stress application through integrins is effective. However, the integrins must be activated and able to induce FA formation in order for mechanical signal conversion to proceed.

Canonically, the cAMP pathway is stimulated at the cell surface through ligand interactions with heterotrimeric G protein-coupled receptors, which typically contain 7 membrane-spanning domains, linked to the Gas subunit. Upon ligand binding to the receptor, conformational changes allow GTP to displace GDP on $G\alpha s$, which can then dissociate from its Gbg binding partner and activate the plasma membrane enzyme adenylate cyclase. Activated adenylate cyclase subsequently catalyzes conversion of ATP to cAMP. Integrins, as heterodimeric receptors, are not conventional G protein-coupled receptors. Nevertheless, we found that activation of cAMP signaling by mechanical stress transfer through integrins can be blocked by the general Ga protein inhibitor, GDP-β-S. However, there has been no demonstration of direct associations between integrin $\beta1$ and $G\alpha s$, the integrin-G protein combination that most likely mediates mechanical activation of cAMP signaling in our studies (9). Thus, since mechanosignaling through cAMP is important for cell and tissue function, and the manner by which mechanically stressed integrins yield increased cAMP remains unknown, a major goal of this proposal was to test the hypothesis that $G\alpha s$ is activated by mechanical stress specifically applied to activated integrin receptors.

Using immunofluorescence microscopy, we found that the heterotrimeric G protein subunits $G\alpha s$ and $G\beta$ congregate with FA proteins (e.g., vinculin, paxillin) at $\beta1$ integrins on human endothelial cells that are both clustered and ligand-activated due to binding to microbeads coated with RGD or activating (BD15) anti-$\beta1$ integrin antibodies, whereas they largely do not codistribute under basal conditions. In contrast, very little recruitment was observed with beads coated with an equal amount of the non-activating K20 antibody or with anti-HLA. Moreover, induction of the recruitment of both G

protein subunits was restored when the K20-bound integrins were subsequently activated by addition of soluble RGD ligand and quantitated using image analysis. Most importantly, application of mechanical force to activated integrins using MTC resulted in additional stress-dependent recruitment of Gαs and Gβ subunits.

Independent confirmation of recruitment of heterotrimeric G proteins to the site of integrin binding was obtained by isolating bead-associated supramolecular complexes, using a modification of our previously published technique for FA enrichment. Cell-bound beads and associated cytoskeletal proteins were dissociated from the rest of the cell by shearing in a mild extraction buffer (0.5% Triton X-100). Western blot analysis of this fraction once again confirmed that Gαs and Gβ proteins were preferentially recruited to the binding sites of RGD and BD15 beads that both ligated and activated surface integrin receptors. This increased recruitment of heterotrimeric G proteins occurred in the presence of equal amounts of β1 integrin binding in the K20, BD15, and RGD conditions, whereas HLA-beads were much less effective, as revealed through Western analysis of the same bead-associated protein fractions.

In order to induce cAMP production, Gαs must be chemically activated, so we employed a novel technique to determine the spatial distribution of G protein activation across the cell. We measured G protein activation directly by quantitating binding of a biotin-labeled, azido-anilido form of GTP (AAGTP) that irreversibly cross-links to G proteins only when they are bound and exposed to ultraviolet (UV) light. Staining for biotinylated AAGTP with fluorescent avidin revealed that low levels of baseline activation of G proteins were observed around RGD- and BD15-beads under non-stressed conditions, however, this activity greatly increased when mechanical stress was applied to those activated integrins compared to controls (HLA, K20). Although a small increase in G protein activation was also observed in cells bound to K20 beads, the most striking and significant population differences arose only when stress was applied via K20 bound integrins that were subsequently ligand-activated by addition of soluble RGD peptide.

To confirm that the biotinylated-GTP was specifically bound to Gαs, cells were separated into cytosolic, cytoskeletal, and bead-associated fractions after UV exposure that were then subjected to gel electrophoresis and probed with avidin-horse radish peroxidase (HRP). The primary GTP binding species in the bead-associated protein fraction displayed a mass of 45 kD and co-migrated precisely with Gαs, as determined by subsequent immunoblotting. Moreover, the observed increase in GTP binding of Gas in the bead fraction required integrin ligation and activation as beads coated with HLA ligands were not effective.

In our most recent studies, we found that beads coated with anti-β1 integrin antibodies also recruited Gαi, Gαq/11, and Gα12, in addition to Gαs, vinculin and talin to the bead-associated complexes. Interestingly, the VEGF receptor also was recruited to the same complexes, even though complexes produced by binding directly to beads coated with anti-VEGF receptor antibodies contained only this receptor and β1 integrin (i.e., they failed to recruit G proteins).

Taken together, these results confirm that even though integrins are not typical G protein-coupled receptors, they can convert mechanical signals into a biochemical response through stress-dependent activation of heterotrimeric Gαs proteins that are adjacent within the FA. These effects could be mediated by direct interactions between integrins and G proteins, or through intermediary partners, such as CD47 or transmembrane growth factor receptors.

The funding provided by this NASA grant also allowed us to make progress in a number of different areas:

- We have completed studies initiated in a past grant period which involved developing a computational model of cell mechanical behavior based on tensegrity. We have demonstrated that this model can predict both the static and dynamic mechanical behaviors of living mammalian cells. In collaboration with Ning Wang (Harvard School of Public Health), we also have developed a new "intracellular stress tomography" technique using the oscillatory form of MTC, and showed that mechanical stresses applied via integrin-bound magnetic beads are transmitted throughout the depth of the cell over discrete cytoskeletal network connections, as predicted by the tensegrity model.

- In a collaboration with Dr. Jing Zhou (Brigham and Women's Hospital), we found that the polycystins 1 and 2, which are encoded by genes that lead to development of polycystic kidney disease when mutated, mediate fluid flow sensation in the primary cilium of renal epithelium and function in the same mechanotransduction pathway. Failure to sense mechanical cues associated with fluid flow due to the lack of functional PC1 or PC2 may therefore contribute to abnormal kidney morphogenesis and development of this disease.

- We performed large-scale magnetic twisting experiments on cultured cells and collected samples that are currently being used for proteomic analysis. The phosphorylation state of 30 different proteins is being analyzed in order to simultaneously determine the mechanosensitivity of many signaling pathways. Similar samples also will be analyzed for genome-wide transcriptional activity using Affymetrix chips.

- We established gene microarray technology in the laboratory, and have used it to identify large classes of mRNAs that specifically associate with the cytoskeleton. Also, to facilitate analysis of genome-wide gene profiling in cells responding to mechanical stress, we developed a Gene Expression Dynamics Inspector (GEDI) that uses self-organizing maps (SOMs) to translate high-dimensional expression profiles of time courses or sample classes into animated, coherent and robust mosaics images. We have used this tool to demonstrate that stable cell phenotypes represent "attractor" states in gene state space. This is important because it provides a conceptual basis to explain how mechanical forces that produce cell distortion can reliably switch cells between the same limited number of cell fates (e.g., growth, differentiation, apoptosis) that specific growth factors induce.

Publications Funded by this Grant:

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Patents Funded by this grant:

Huang S & Ingber DE, inventors. "Methods for Analyzing Dynamic Changes in Cellular Informatics and Uses Therefor " (pending)

Huang S, Eicher G, & Ingber DE. "Method and Apparatus for Displaying Large Amounts of Information" (pending)