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Using Magnets and Magnetic Beads to Dissect Signaling Pathways Activated by Mechanical Tension Applied to Cells

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Abstract

Cellular tension has implications in normal biology and pathology. Membrane adhesion receptors serve as conduits for mechanotransduction that lead to cellular responses. Ligand-conjugated magnetic beads are a useful tool in the study of how cells sense and respond to tension. Here we detail methods for their use in applying tension to cells and strategies for analyzing the results. We demonstrate the methods by analyzing mechanotransduction through VE-cadherin on endothelial cells using both permanent magnets and magnetic tweezers.

Keywords

Mechanotransduction; tension; VE-cadherin; endothelial cell

1. Introduction

Analyses of physical forces applied to or produced within tissues and their molecular responses at the cellular level (i.e. mechanotransduction) have become important due to the role force-sensing has in normal biology and disease. For instance, endothelial cells of the vasculature experience shear and pulsatile forces under normal conditions as well as environmental stiffening upon progression of atherosclerosis. Cells detect these forces through cell-cell and cell-extracellular matrix (ECM) interactions, where cell-surface adhesive receptors form the links between neighboring cells or between cells and the ECM [1]. Adhesion receptors form the physical connection between the intracellular actin cytoskeleton and the surrounding environment, and some of these receptors sense tension differences and transduce this fluctuation into a chemical signal, such as activation of Rho

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GTPase signaling pathways [2]. How extracellular tension regulates cellular responses through cell-cell adhesion receptors is an important question in normal biology and disease.

Cadherins are a family of cell-cell adhesion receptors that are major components of adherens junctions and have been associated with tension sensing in cells [1]. Vascular endothelial-cadherin (VE-cadherin) is a classic cadherin mainly expressed on the plasma membrane of endothelial cells that line the luminal surface of blood vessels [3]. The initial discovery of VE-cadherin showed that this cadherin was involved with the barrier function of the endothelial layer by controlling permeability [4]. VE-cadherin at cell-cell junctions becomes disorganized during leukocyte trafficking between neighboring endothelial cells [5]. Tumor cells also induce disruption of the VE-cadherin contacts [6,7]. Under fluid shear stress, endothelial cells respond to the force through a mechanosensory complex involving VE-cadherin [8]. Under disease states, such as atherosclerosis, the physical environment of the endothelial cells changes and so does their response to external forces [9]. Coon and colleagues showed that the transmembrane domain of VE-cadherin serves an important role associating with VEGFR2/3 to form a mechanosensory complex in endothelial cells [10]. These data suggest that VE-cadherin has essential functions in mechanotransduction in endothelial cells to allow for responses to external forces from the extracellular environment.

Various tools have been utilized to study the effects of external forces on cells, including atomic force microscopy (AFM), optical tweezers, flow systems, PDMS microneedle substrates, and FRET tension sensors [11,12]. However, biochemical analysis is difficult with many of these techniques, whereas the use of magnetic beads to apply tension to a plate of cells readily facilitates biochemical assays. Magnetic beads also permit single cell assays, such as the measurement of bead displacements in response to repeated pulses of applied force using magnetic tweezers. The ability to perform both single cell assays as well as bulk biochemical assays makes magnetic beads a valuable tool in the study of mechanotransduction, since this is not possible using approaches that can only analyze single cells such as optical tweezers or AFM. Another benefit is the ability to apply the force to a targeted receptor unlike more general force analysis tools (i.e. flow or traction force systems). Lastly, magnetic tweezers provide a much larger dynamic range of force application when compared to optical tweezers and AFM; they are able to apply weak forces (5 pN) similar to optical tweezers as well as strong forces (1 nN) on the same level as AFM [13]. In biology, cells are exposed to different types of force. Some forces are acute, some are sustained, some build slowly over time, and others are cyclical with periods of tension followed by relaxation. The different techniques all have advantages. The magnetic beads as used in assays by us mimic biological situations where force is applied relatively quickly for a short sustained period (permanent magnet) or a regimen of brief pulses of force with intervening periods of relaxation (magnetic tweezers). It should be noted that these force applications do differ slightly and can produce different cellular responses as was shown by the Fredberg group where they showed cellular reinforcement (stiffening) or fluidization (softening) was dependent on the force regime (frequency, amplitude) applied to the cell [14,15]. For these reasons, our lab utilizes magnetic beads for broad biochemical analyses using permanent magnets as well as fine tuned magnetic tweezers for pulling experiments to measure the stiffening response of cells to applied forces (Figure 1).

Several different studies have used magnetic beads to explore how cells respond to mechanical forces exerted on cell adhesion molecules. In early work, Wang *et al.* used RGD-coated magnetic beads to apply a twisting force to integrins on the surface of endothelial cells and observed a stiffening response that was dependent on the actin cytoskeleton [16]. McCulloch's group used permanent magnets placed above cell cultures to pull vertically on collagen-coated magnetic beads adhering to the dorsal surface of cells. This allowed them to perform both single cell analysis, measuring for example increases in intracellular calcium in response to force, as well as bulk biochemical measurements on large populations of cells, such as analyzing protein tyrosine phosphorylation, which they showed increased in response to force [17,18]. In subsequent work the same group used this approach to show that sustained tension on integrins via magnetic beads coated with collagen activated RhoA [19]. Ingber and his colleagues used magnetic tweezers to examine the effects of applying tension on magnetic beads coated with integrin ligands and implicated RhoA signaling pathways in the cellular response [20]. Na *et al.* used the combination of FRET and magnetic twisting cytometry (MTC) to analyze rapid mechanochemical signaling in live cells and showed the prestressed cytoskeleton promoted rapid activation of Src upon force application [21,22]. Using this approach Poh *et al.* also showed that force application through integrins activated Rac1 and was independent of Src activity in human airway smooth muscle cells [23].

Following on from these studies, our lab has combined both biochemical analyses using permanent magnets with single cell experiments using magnetic tweezers to analyze the signaling pathways downstream from tension applied to integrins [24]. We used fibronectin-coated beads to pull on fibroblast integrins and showed the activation of RhoA was mediated by two distinct pathways that activate the Rho GEFs, LARG and GEF-H1. Additionally, activation of RhoA via these GEFs contributed to the observed cellular stiffening [24]. Magnetic beads and magnets have been used to apply force to other cell adhesion molecules. For example, Tzima's lab have shown that tension applied to PECAM-1, an endothelial cell adhesion molecule implicated in endothelial mechanotransduction, activates RhoA in an integrin-dependent pathway via GEF-H1 and LARG [25]. We showed that tension on ICAM-1 on endothelial cells causes cell stiffening and helps mediate transendothelial migration of leukocytes [26]. In another study, DeMali's lab used a similar approach to exert force on E-cadherin using magnetic beads coated with the extracellular domain of E-cadherin. They discovered that the tension-induced recruitment of vinculin depended on the phosphorylation of vinculin at Y822 [27]. Another study by Kim *et al.* used E-cadherin-coated magnetic beads to show α -catenin is an integral part of the force sensing apparatus at cell-cell junctions [28]. Other labs have also shown that vinculin, α -catenin, and actin are recruited to E-cadherin adhesions in response to force [29-31]

The versatility of using magnetic beads to generate tension is illustrated in a study in which the response of an organelle, the nucleus, to tension was examined. In this work, tension was applied to isolated nuclei using magnetic beads coated with antibodies against the nuclear envelope protein nesprin-1. Unexpectedly, successive applications of force resulted in decreased bead displacement implying that the isolated nuclei were stiffening [32]. Together

these studies demonstrate the usefulness of magnetic beads in mechanotransduction experiments.

Here we detail the methods for using magnetic beads to apply forces to surface proteins. We focus on force application to the cell adhesion receptor VE-cadherin on endothelial cells, and show that tension on VE-cadherin stimulates mechanotransduction via Rho GTPase signaling and alters protein tyrosine phosphorylation.

2. Materials and Methods

2.1 Materials

The superparamagnetic beads (particles that magnetize upon placement in a magnetic field but lose magnetization upon removal from the field) we utilize for these assays were 2.8 μm diameter tosyl-activated magnetic beads from Invitrogen (Dynabeads M-280 Tosyl-activated; Cat. #142.03) or Dynabeads of 4.5 μm diameter (Cat. #140.13) if larger forces and surface contacts are needed. Common chemicals and experimental reagents were from Sigma Aldrich and Fisher Scientific. A list of some of the other materials used:

EBM2 medium (Lonza)

EGM2 SingleQuots (Lonza)

Delipidated BSA (Sigma)

hVEC-Fc (Sino Biological)

Dynal Magnetic Particle Concentrator MPC-S (Invitrogen; Cat. #120.20)

Colloidal Blue Stain (Invitrogen)

Neodymium magnets, 3" \times 1/2" disc, NdFeB – grade N52 (K&J Magnetics, Inc.)

Neodymium magnets, 5/8" \times 1/4" disc, NdFeB – grade N52 (K&J Magnetics, Inc.)

10 cm plastic cell culture dishes (Costar)

VE-cadherin antibody (Santa Cruz, F-8)

Phosphotyrosine antibody (Millipore, 4G10)

Actin antibody (Sigma)

α -catenin antibody (BD)

cell scraper, 25 cm (Sarstedt)

PBS, pH 7.6 without Ca^{2+} or Mg^{2+} (Invitrogen)

Coverslips (Corning): Square; No. 1; Material: borosilicate glass; Thickness: 0.12 to 0.16mm; Size: 22 \times 22mm.

Rectangle; No. 1.5; Material: borosilicate glass; Size: 24 \times 50mm Clear nail polish

Microscope slide (Fisher Scientific)

Vacuum grease (Fisher Scientific)

Cloning rings (Fisher Scientific)

We obtained pooled-donor primary human umbilical vein endothelial cells (HUVECs) from Lonza and cultured them in EGM2 medium up to passage 10. For experiments, HUVECs were grown to 80-100% confluent monolayers.

2.2 Ligand Conjugation to Magnetic Beads

Ligands for targeting cell surface adhesion receptors for mechanotransduction analyses can be covalently linked to the superparamagnetic beads of 2.8 μm or 4.5 μm diameter. Bead diameter should be restricted to 2-5 μm since smaller beads tend to more quickly undergo phagocytosis during 30-60 minute incubation and larger beads have stronger adhesion to the cell and would restrict bead displacement under the utilized magnetic field strengths [33-35]. Limiting bead size and incubation time with the cells will help avoid these problems. Here we use the human VE-cadherin extracellular domain fused at the C-terminus with the Fc domain of IgG₁ (hVEC-Fc) to target cellular VE-cadherin molecules for force application. The conjugated ligand does not have to be a physiological ligand like the extracellular domain of a cadherin, it could also be a monoclonal antibody targeting a cell surface protein, such as an antibody specific for a MHC class I receptor. We covalently cross-linked hVEC-Fc to 2.8 or 4.5 μm diameter magnetic beads (Figure 2A) in the following procedure:

1. Prepare buffers as per Invitrogen Dynabead M-280 Tosyl-activated protocol:
 - a. Buffer B – 0.1 M Sodium Phosphate Buffer, pH 7.4
 - b. Buffer D – 0.01 M Sodium Phosphate, 0.0137 M NaCl, and 0.5% (w/v) delipidated BSA, pH 7.4
 - c. PBS
2. Resuspend lyophilized hVEC-Fc in sterile PBS to 250 $\mu\text{g}/\text{mL}$, divide into 100 μL aliquots, and store at $-80\text{ }^{\circ}\text{C}$.
3. Wash 82.5 μL (6×10^8 beads) of the 2.8 μm tosyl-activated Dynal beads in 1 mL Buffer B in 1.5 mL microcentrifuge tube and use the Dynal magnetic particle concentrator (MPC) to pellet beads and aspirate the buffer.
4. Combine 20-25 μg hVEC-Fc (80-100 μL) with appropriate volume of Buffer B to bring the total volume to 200 μL and mix by pipetting. [For gel analysis take 10 μL aliquot for crosslinking analysis and mix with 10 μL 2X Laemmli Sample Buffer (Input); Figure 2B]
NOTE: BSA or poly-lysine coated beads can be used as negative controls and produced in a similar manner.
5. Combine hVEC-Fc with beads, transfer to 0.5 mL microcentrifuge tube, and incubate beads with hVEC-Fc for 18-24 hours at $37\text{ }^{\circ}\text{C}$ on a rotor to allow for the reaction to occur and produce a covalent linkage of protein to the bead (Figure 2A).
6. Pellet beads using the MPC and remove 10 μL aliquot and mix with equal volume of 2X; Laemmli Sample Buffer (Output), Figure 2B.

7. Wash beads 3 times with 1 mL PBS using MPC to pellet the beads. Use the first 1 mL PBS to transfer the beads back to a 1.5 mL microcentrifuge tube.
8. Resuspend beads in 1 mL PBS without Ca^{2+} (removal of calcium ions blocks the homophilic interaction of VE-cadherin to prevent bead aggregation) to give a concentration of 6×10^8 beads/mL.
9. Store beads at 4 °C for up to 3 months.
10. (Optional) Run a SDS-PAGE gel and stain with colloidal blue to analyze the hVEC-Fc crosslinking to the magnetic beads (Figure 2B).
11. (Optional) Determine the force applied to a single bead by the magnet.
 - a. For the permanent magnet, we create a chamber slide using a standard microscope slide, coverslips, and nail polish to glue them in place (Figure 2C) and measure the distance the bead front moves through a channel 10 mm long and containing undiluted glycerol, a Newtonian fluid of known viscosity ($\eta = 1.41$ Pa-sec at 20 °C). The well where the magnetic beads are placed (5 μL of 1:10 dilution of beads in glycerol) is 10 mm from the edge of the slide and provides a fixed distance between the magnet and the well. The magnet is then brought into perpendicular contact with the slide edge mimicking the experimental distance. Measurements of how far the beads travel towards the magnet are taken at two time points, 15 and 30 minutes (At 30 min the 4.5 μm beads reached ~ 6 mm from the magnet). Ideally, the 6 mm mark should be the center of the distance travelled for a more accurate force estimate, but cutting the glass coverslip evenly at smaller widths is difficult and often not practical. From these empirical measurements the average rate of the bead is obtained and can be used to calculate an approximate force exerted by the magnet at this fixed distance used in the experiments (6 mm). The Stokes law equation used is

$$F = 6 \pi r_{\text{bead}} \eta v_{\text{bead}}$$

Where F is the force generated by the magnet on the beads, r_{bead} is the radius of the bead, η is the viscosity of the Newtonian liquid, and v_{bead} is the measured velocity of the bead. Using this method for our permanent magnet system, we have calculated the force to be ~ 40 pN on the 2.8 μm bead and ~ 200 pN on the 4.5 μm bead.

- b. For the magnetic tweezers, this same equation is used to calculate the force applied by the magnetic pole tip on a 2.8 μm bead at a distance of 20-30 μm in another Newtonian fluid, Karo syrup ($\eta = 3.4$ Pa-sec at 20 °C). Using the same experimental force regimen, we measure the distance the bead moves and determine the rate. The force the pole tip applies with these parameters is about 20–40 pN.

2.3 Application of tension to cells through surface receptors using ligand-coated magnetic beads and permanent magnets for biochemical analyses (e.g. RhoGTPase activation, phosphotyrosine, and adhesion complex analyses)

A superparamagnetic bead coated with a specific ligand for a cell surface receptor is a useful tool for applying force to that specific receptor when placed within a magnetic field gradient. By placing the cells with adherent magnetic beads within a magnetic field of a permanent magnet, a defined force can be applied to the cell. After this treatment the cells can be lysed and processed for biochemical analyses (Figure 3A).

A permanent magnet (we use Neodymium magnets which are 10 times stronger than ceramic magnets) is used to generate perpendicular, tensile forces on magnetic beads adhering to the apical surface of cells. For all experiments, the pole face of the magnet is parallel with the 10 cm culture dish surface at a height of 6 mm. At this distance the force on a single 2.8 μm magnetic bead is ~ 40 pN and on a single 4.5 μm magnetic bead is ~ 200 pN. A constant force of varying duration is used for all experiments.

1. Prepare buffers:
 - a. Lysis Buffer – 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 % Triton-X100, 10mM MgCl_2 , 1 mM Sodium orthovanadate, and protease inhibitors
 - b. Serum-Free Medium (SFM) – EBM2 medium and 0.25% (w/v) delipidated BSA
 - c. PBS without Ca^{2+} or Mg^{2+} , pH 7.6
2. Culture HUVECs on 10 cm tissue culture dishes in EGM2 medium until an 80-100% confluent monolayer of cells is formed.
3. Aspirate the growth media and add 5 mL of warmed PBS without Ca^{2+} or Mg^{2+} per dish for 2-5 minutes to perform a mild calcium switch, which disassociates cadherin interactions to free up receptors for bead ligation. (For cell-cell junction receptors like cadherins that require Ca^{2+} for binding, a calcium switch can be performed to temporarily dissociate cell-cell junctions and free up receptors to interact with ligand coated beads. By adding PBS (phosphate can extract Ca^{2+} from weaker cell-cell junctions like endothelial cells) or 4 mM EGTA in PBS (EGTA chelates Ca^{2+} and is more stringent for tighter cell-cell contacts like epithelial cells) for 5-60 minutes.
4. Aspirate the PBS and add 3 mL of SFM per 10 cm dish.
5. Vortex hVEC-Fc magnetic beads for 30-60 seconds and add predetermined volume to appropriate dishes and promptly rock the dish back and forth to disperse the beads evenly in the medium and across the dish which will prevent aggregation of the beads. (Brief, low-level sonication prior to addition to the cells may be used if the beads tend to aggregate.)
 - a. For 2.8 μm beads, add 100 μL hVEC-Fc beads (60×10^6 beads) per dish.
 - b. For 4.5 μm beads, add 150 μL hVEC-Fc beads (7.5×10^6 beads) per dish.

NOTE: A confluent monolayer of HUVECs on a 10 cm dish has about 6×10^6 cells, so the bead addition is 1 bead per cell. More beads can be used if needed but bead aggregates start to form.

6. Allow the beads to settle and adhere to the cells for 15-30 minutes under cell culture conditions (37°C and 5% CO₂). Bead adhesion to cells can be determined by observing the beads under a light microscope using a 20X objective.
7. For treatments using magnets, normal dish lids are exchanged for lids containing 3 inch diameter magnets held in place by 2 smaller 5/8 inch diameter magnets (Figure 3A) and incubated for the designated time points at cell culture conditions or room temperature [17,24].

NOTE: These magnets are extremely strong and special attention needs to be taken to avoid personal injury or equipment damage. A designated area and spatial distance between individual magnets needs to be maintained.

8. Remove magnet lids, replace original lid, and place dishes on ice. **NOTE:** All handling of samples from here on is done on ice at 4°C.
9. Aspirate medium from the dishes. (Tilting dishes for 30 seconds to remove remaining medium helps to keep total lysate volumes equal.)
10. Add 500 µL of ice-cold lysis buffer per dish, scrape the lysate from each dish with a cell scraper, and transfer the lysates to 1.5 mL microcentrifuge tubes.
11. Pellet the magnetic beads using the MPC and transfer lysates to new 1.5 mL microcentrifuge tubes. (For analysis of associated adhesion complexes, wash the magnetic beads 3 times with 1 mL of lysis buffer. Add 50 µL 2X Laemmli Sample Buffer to beads, boil tubes, and store at -20°C.) **NOTE:** One way to normalize data from these samples since pulling can cause noticeable bead loss is to count the beads during the last washing step using a hemacytometer or cellometer. The ratios of magnet-treated sample bead number over the no-magnet control bead number can be used to normalize the volume of samples loaded onto gels for SDS-PAGE and western blot analysis.
12. Pellet the insoluble fraction in the lysates by using a microcentrifuge at 16,000 g for 5 minutes at 4°C and transfer lysates to new tubes. (The insoluble pellet can be washed with lysis buffer and resuspended in 2X Laemmli Sample Buffer for analysis of the TX;100 insoluble cell fraction).
13. Remove a 50 µL aliquot from each lysate and add to new tubes containing an equal volume of 2X Laemmli Sample Buffer, boil the tubes, and store at -20°C. (These samples can be used for total protein controls, phosphoprotein analysis, soluble versus insoluble fraction analysis, etc.)
14. The remaining 450 µL of lysate can be used for pull down experiments such as Rho GTPase activation assays, immunoprecipitation experiments, or other biochemical assays. We utilize the lysates for Rho GTPase activity assays as described previously [36].

2.4 Magnetic tweezer application of pulsed forces for measuring cell stiffening

Magnetic tweezers can be used to apply pN tensional force to magnetic beads bound to cell surface receptors. When coupled to live cell imaging, this experimental system allows the measurement of local viscoelastic properties by tracking the bead displacement due to a known force generated by the magnetic field from an electromagnetic pole tip. We used the 3-dimensional force microscope (3DFM) designed by the Center for Computer Integrated Systems for Microscopy (<http://cisimm.cs.unc.edu>) to apply tension to various cell surface receptors, including integrin [24], ICAM-1 [26] or as we show here, VE-cadherin. The 3DFM is composed of thin and flat magnetic poles mounted on an Olympus IX81-ZDC2 inverted microscope (Olympus) equipped with a 40× objective (Olympus UplanLN 40×/0.75) and a highspeed Rolera EM-C2 camera (QImaging) to record bead movement. Description and calibration of the 3DFM system has been detailed previously [37]. Here we describe how to use ligand-coated magnetic beads in this experimental system to measure endothelial cell mechanical properties in response to tension applied to VE-cadherin.

1. Culture HUVECs in EGM2 medium on sterile glass coverslips (24 × 50 mm) coated with collagen (10 µg/ml) within a cloning ring. Grease, such as silicon vacuum grease is applied around the cloning ring to prevent medium leakage. Cells are cultured until they reach 80-100% confluence for at least 24 hours.
2. Aspirate the growth media and add 500 µL of warmed PBS per dish for 5 minutes.
3. Remove the PBS and add 500 µL of SFM.
4. Vortex 2.8 µm hVEC-Fc beads for 1 min (see hVEC-Fc beads preparation in 2.2) and add 10³ beads per cloning ring. Allow the beads to adhere for 30 minutes under cell culture conditions (37°C and 5% CO₂). NOTE: Other bead sizes can be selected (from 1 to 4.5 µm) to generate different amounts of force. For example, 4.5 µm beads can be used to apply up to 10 nN force.
5. Remove the medium and replace with SFM. Place the coverslip on the microscope stage, remove the cloning ring and approach the magnetic pole to the coverslip surface (at approximately 80 µm above the cell surface to avoid any damage to the cells).
6. Select a cell that has only one bead bound to its surface and position the tip 20–30 µm away from bead. NOTE: the distance between the tip and the bead can be adjusted depending on magnetic pole calibration, with our system a distance of 20-30 µm yields a 20–40 pN force (See section 2.2).
7. Eliminate any remnant magnetization from the pole tips (activation of the degauss mode as detailed previously [38]), start the force protocol, and record bead movement at 30 frames/second. NOTE: Typical force protocol generates 10 cycles of 4 sec extension (force on ~20–40 pN) and 3 sec recovery (force off).
8. Eliminate any remnant magnetization from the pole tips before moving the magnetic pole within the sample.
9. Repeat steps 6 and 7.

Measure and analyze the bead displacement. Changes in the mechanical properties of the cells in response to force application can be determined by measuring the bead displacement for each pulse. For example, a decrease in bead displacement will indicate an increase in cellular stiffness, as described previously when forces are applied to integrin-based adhesion [24]. Measurement of the bead displacement (micrometer) can be performed using Video Spot Tracker (software designed by the Center for Computer Integrated Systems for Microscopy and Manipulation -<http://cismm.cs.unc.edu>). Relative bead displacement can be calculated by normalizing the displacement for each pulse to that observed during the first pulse. **NOTE:** Regarding statistical analysis, beads that show displacements of less than 10 nm (detection resolution) and loosely bound beads are excluded from analysis. Depending on the sample size (n =number of beads), parametric (ANOVA) or non-parametric (Kruskal-Wallis) statistical tests can be used to analyze bead displacement. To quantify the change in local stiffness, the spring constant (Pa) can be calculated for each pulse by fitting the bead displacement and force magnitude to a modified Kelvin–Voigt model [39] for a viscoelastic liquid.

3. Results and Discussion

3.1 Human VE-cadherin extracellular domain/Fc fusion protein (hVEC/Fc) crosslinking to 4.5 μ m tosyl-activated magnetic beads

We covalently bonded hVEC-Fc to 4.5 μ m tosyl-activated magnetic beads to provide a more stable ligand for force application to cellular VE-cadherins. In Figure 2A, the reaction between the tosyl groups of the magnetic beads and the primary amines of hVEC-Fc is depicted. The end result is a ligand covalently bonded to a magnetic bead, which is a useful tool for force application experiments since the ligand is less likely to dissociate from the bead during force application. To confirm the adsorption of hVEC-Fc onto the tosyl-activated magnetic beads, we analyzed 10 μ L aliquots (\sim 2 μ g of protein) of the hVEC-Fc protein input (IN) and output (OUT) from the reaction mixture by SDS-PAGE followed by colloidal blue staining (Figure 2B). A 120 kDa hVEC-Fc protein band of about 2 μ g (determined by BSA loading controls) is evident in the input lane and absent in the output lane indicating that the hVEC-Fc protein has been covalently bound to the surface of the bead. This data shows that the beads are ready for use in force application experiments.

3.2 Biochemical analyses of mechanical tension applied to VE-cadherin on HUVECs by hVEC/Fc-coated magnetic beads

We utilized the 4.5 μ m, hVEC-Fc-coated magnetic beads to apply mechanical tension to VE-cadherin on the surface of HUVECs by using a permanent magnet set-up depicted in Figure 3A. This procedure was used to carry out several biochemical assays to determine how HUVECs respond to forces on VE-cadherin. Rho GTPases are master regulators of the cellular cytoskeleton, and their activities are important in mechanotransduction. For this reason, we measured activation levels of two Rho GTPases, RhoA and Rac1, after 1 or 5 minutes of force application on VE-cadherin (Figure 3B). RhoA activity increased significantly by 5 minutes, whereas Rac1 showed decreased activation by 5 minutes. This data supports that HUVECs respond to mechanical tension on VE-cadherin by activating

RhoA and inhibiting Rac1 activity potentially through crosstalk in a similar tug-of-war mechanism as shown previously [40,41].

Given that cell-cell junctions are prominent sites of tyrosine phosphorylation, we examined whether mechanical tension on VE-cadherin affected the level of protein tyrosine phosphorylation (Figure 3C). The hVEC-Fc magnetic beads and a permanent magnet were used to apply force on HUVECs as above. Cell lysates were prepared at varying times and the levels of protein tyrosine phosphorylation (pTyr) were determined by Western blot using a phosphotyrosine-specific antibody (4G10). Compared to the control lane, there were several protein bands between 150-25 kDa that changed pTyr levels with the addition of hVEC-Fc beads as well as when tension was applied to the beads (arrows signify protein bands of interest). Simple engagement of the hVEC-Fc with VE-cadherin elevated pTyr levels. This was a surprising finding, but the recent report that VE-cadherin associates with VEGFR2/3 through the transmembrane domain might explain this observation [10]. The hVEC beads can cause clustering of these membrane proteins that would lead to increases in VEGFR signaling and tyrosine phosphorylation. Whereas tension on VE-cadherin decreased the level of pTyr to an intermediate value or to the resting level. A band of ~25 kDa shows an increase in pTyr levels with force application relative to control beads and beads alone. Further experiments are needed to identify these pTyr proteins and whether tension is broadly activating protein tyrosine phosphatases or inhibiting protein tyrosine kinases, or both.

The last biochemical analysis we utilized was to identify components of the adhesion complex in HUVECs that are isolated with the hVEC-Fc magnetic beads (Figure 3D). Endogenous VE-cadherin was detected using an antibody that only recognizes a site in the cytoplasmic tail of VE-cadherin, which is not present in the hVEC-Fc recombinant protein. Importantly, we observed VE-cadherin in all treatments confirming an association between cellular VE-cadherin and the hVEC-Fc magnetic beads. Interestingly, a slight increase in VE-cadherin recruitment was detected with 5 minutes of tension. Several cellular proteins are known to associate with the cytoplasmic tail of VE-cadherin to form an adhesion complex including α -catenin and actin. We detected both of these proteins within the adhesive complex isolates from all treatments. However, a small increase in α -catenin levels with 5 minutes of tension was detected that is consistent with previous studies showing that tension on VE-cadherin recruits cellular components of the VE-cadherin adhesion complex [42,43]. Quantification of the α -catenin to VE-cadherin protein bands was done to determine if the increase in α -catenin was independent of increasing VE-cadherin (Figure 3D, graph). The analysis shows a trend that α -catenin increases relative to the amount of VE-cadherin but it was not statistically significant ($p = 0.1$). The possible increase in α -catenin is likely dependent on both force and increased VE-cadherin presence.

3.3 Cellular stiffening of HUVECs pulled on by hVEC/Fc magnetic beads using magnetic tweezers

To investigate how cells adapt their mechanical properties in response to tension applied to VE-cadherin, we used 2.8 μm hVEC-Fc-coated magnetic beads bound to HUVECs. We observed typical viscoelastic displacement for each pulse of tensional force (Figure 4A). We

found that progressive application of pulses of constant force induced a significant decrease in cellular strain (Figure 4B), indicating local cellular stiffening. This result signifies that tension applied to VE-cadherin triggers adhesion remodeling, which produces a local stiffening. This is consistent with work from others demonstrating that mechanical tension regulates VE-cadherin-based adhesion growth [44,45]. Liu *et al.* showed that endothelial cell-cell adhesion size is regulated by tugging forces through balancing myosin and Rac1 activities [44]. Interestingly, Barry *et al.* showed that externally applied tension triggers vinculin recruitment to VE-cadherin [45]. Vinculin is recruited by VE-cadherin to a subset of endothelial cell-cell junctions called focal adherens junctions (FAJs) that undergo remodeling upon increased cellular tension [42]. Tension-dependent recruitment was similarly shown for E-cadherin [29]. Since we observed that application of tension to VE-cadherin activates RhoA and stimulates the recruitment of α -catenin (Figure 3B and 3D), it will be interesting to test the involvement of these proteins using the magnetic tweezers. Identifying the molecular events, which regulate this mechanical response may help to understand how mechanical stress controls endothelial functions.

4. Conclusions

Ligand-coated magnetic beads are a very useful tool for measuring cellular tension since they can be used in diverse analyses. We describe here their application in tension assays using permanent magnets in biochemical readouts as well as magnetic tweezers to examine the response of single cells to pulses of force. We have illustrated these applications of magnetic beads to analyze the effects of tension on VE-cadherin on endothelial cells. We have shown that force applied to VE-cadherin causes an increase in RhoA activation with a correlative decrease in Rac1 activation, a change in protein tyrosine phosphorylation levels, as well as a stiffening response to multiple short pulses of force. These are just some of the assays the magnetic beads can be used for, but other types of biochemical analysis are possible (e.g. other signaling pathways, mass spectrometry, etc.), as well as using the beads and the permanent magnet system beneath the culture dish to apply compressive forces to cells.

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Highlights

- Detailed procedures for use of ligand-coated magnetic beads for force application to cells using biochemical analyses and magnetic tweezers.
- As an example, we show tension applied to VE-cadherin on endothelial cells increases RhoA activation with corresponding decrease in Rac1 activation, changes in phosphotyrosine levels, and increased recruitment of adhesion complex proteins.
- Pulsed force application to VE-cadherin produces cellular stiffening.

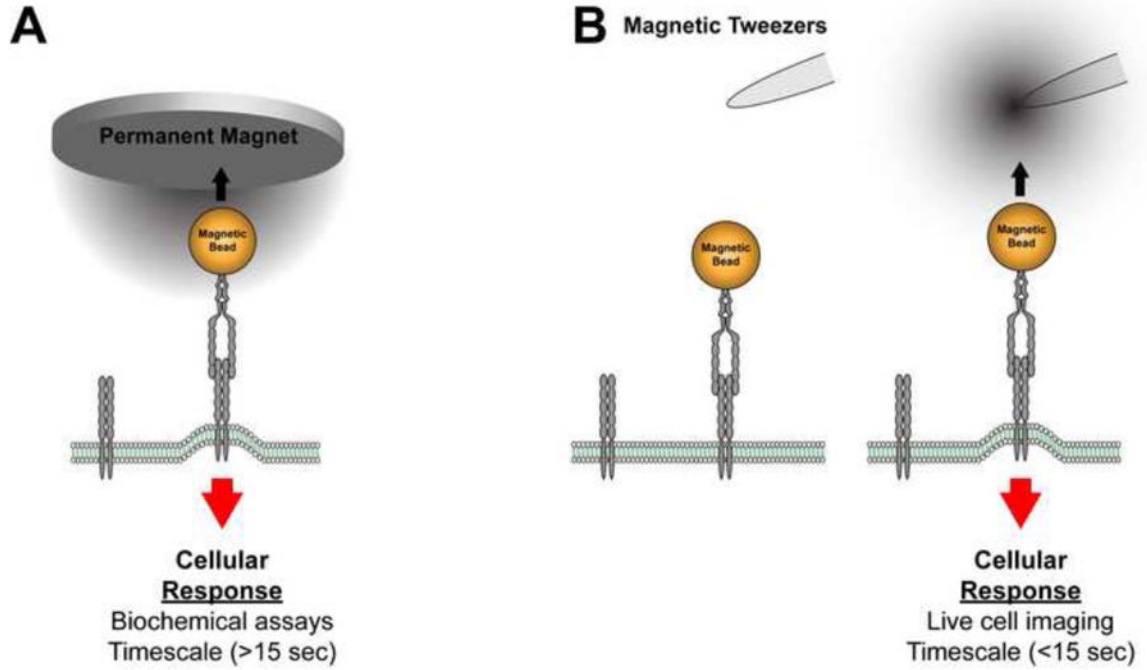


Figure 1. Use of protein-coated magnetic beads in cell surface receptor tension experiments
A, Permanent magnets can be used to apply pulling forces to ligand-coated magnetic beads adhered to cell surface receptors. Cell responses to tension on the timescale of 15 seconds and greater can be analyzed by various biochemical assays using this method. B, Magnetic tweezers can utilize ligand-coated magnetic beads to probe tension responses by individual cells on shorter timescales than permanent magnets and measure real time responses using live cell imaging.

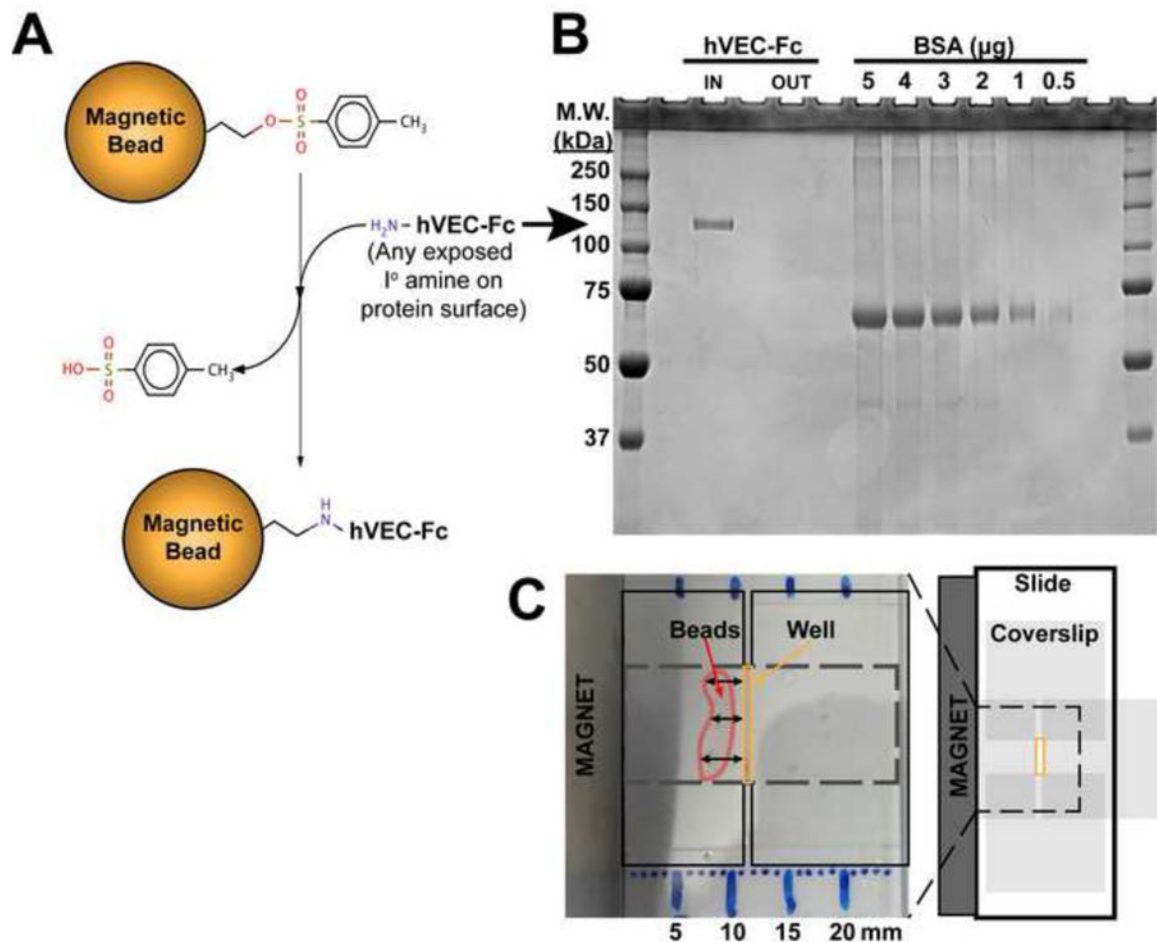


Figure 2. Preparation of hVEC-Fc magnetic beads

A, Tosyl-activated magnetic beads allow for chemical crosslinking of the ligand to the beads. **B**, Colloidal blue staining of SDS-PAGE analysis of hVEC-Fc conjugation to Dynal magnetic beads. The reaction input lane (IN) shows a protein band of molecular weight corresponding to hVEC-Fc (~120 kDa) and amount (~2 μg) was present prior to conjugation. The reaction output lane shows a loss of the hVEC-Fc band from the solution indicating the protein was conjugated to the beads. **C**, Chamber slide set-up for obtaining a coarse measurement of the amount of force applied to a single magnetic bead by a permanent magnet. Using a microscope slide, two square coverslips were cemented to the slide forming a 10 mm channel. Glycerol was pipetted onto the slide to fill the channel (50 μL). A third coverslip was scored and broken in half to form the ceiling of the chamber through which the beads will run. A fourth square cover slip was used to cover the remaining channel and create a well in between which 5 μL of a 1:10 dilution of beads in glycerol were loaded and the chamber closed and allowed to equilibrate for 5 minutes before the magnet was applied perpendicularly to the side of the slide as indicated. Measurements of how far the bead front migrated at two time points (15 and 30 minutes) were taken at three points and used to calculate the mean bead velocity (v). This variable was then used to calculate the force of the magnet.

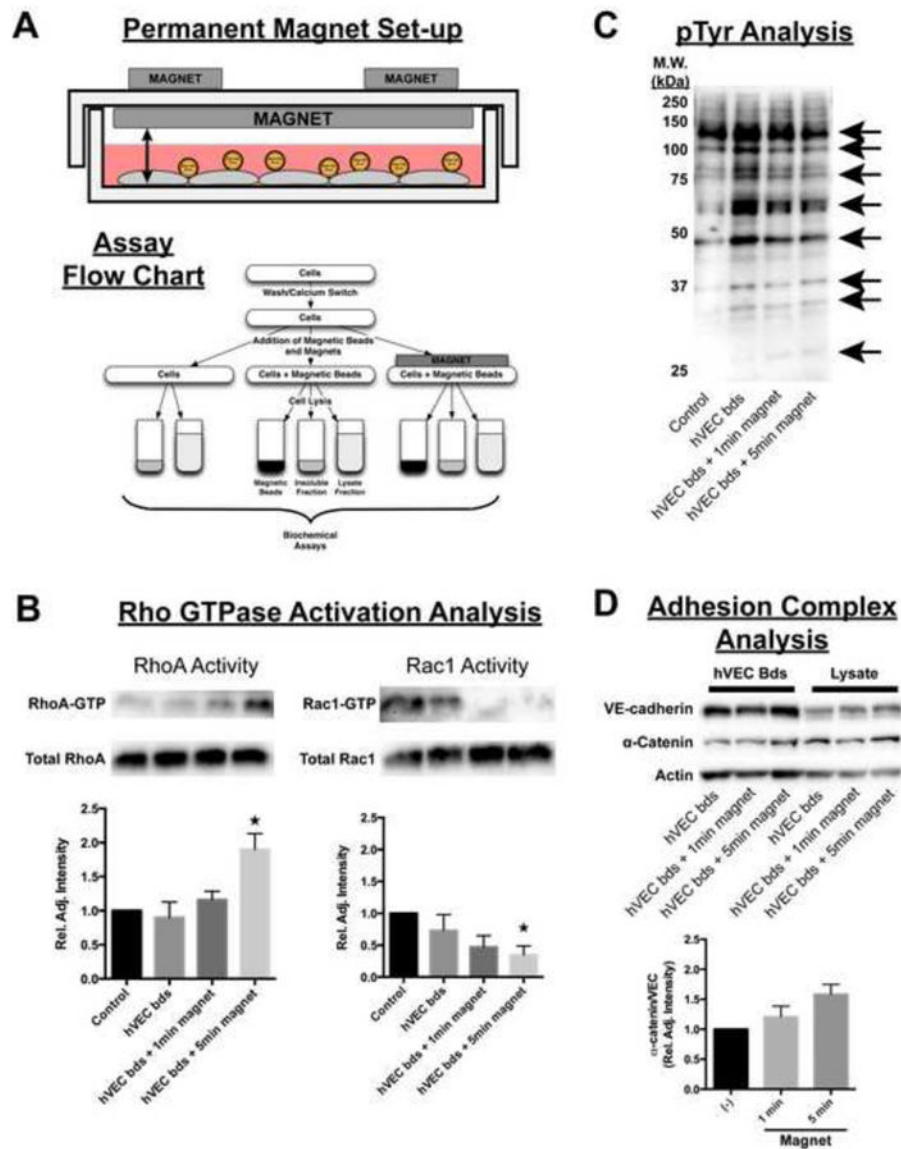


Figure 3. Permanent magnet force application and biochemical assays for Rho GTPase activation, protein phosphotyrosine levels, and adhesion complex analysis

A, A diagram of the permanent magnet setup on the lid of 10 cm culture dish and a general flowchart of the permanent magnet assay. The position of the magnet places the face of the magnet about 6 mm from the bottom of the dish (arrow), which fixes the distance of the magnet from the cells, makes the field strength constant, and permits control of the time of exposure to the magnetic field. **B**, RhoA and Rac1 activity assays were done on untreated control cell lysates or lysates from cells treated with hVEC-Fc beads with no exposure to the permanent magnet or 1 or 5 minutes of magnet exposure. Western blots of RBD-pulldowns (activated RhoA) or PBD-pulldowns (activated Rac1) and cell lysates for total protein are shown. Quantification of protein bands was done by densitometry and results are displayed in the graphs ($n=4$; $p < 0.05$). **C**, Western blot analysis for protein phosphotyrosine levels in cell lysates that were treated as above. Arrows indicate protein bands of interest that show changes in phosphotyrosine levels when compared to the control lane. **D**, Western blot

analysis of VE-cadherin adhesion complex proteins associated with the hVEC-Fc magnetic beads from treatments with hVEC-Fc beads with no exposure to the permanent magnet or 1 or 5 minutes of magnet exposure. Cell lysates from these treatments were also analyzed. VE-cadherin, α -catenin, and actin were detected. The graph shows quantification of the α -catenin and VE-cadherin protein bands pulled down by the magnetic beads and plotted as the ratio of α -catenin/VE-cadherin, mean \pm SEM for n=3.

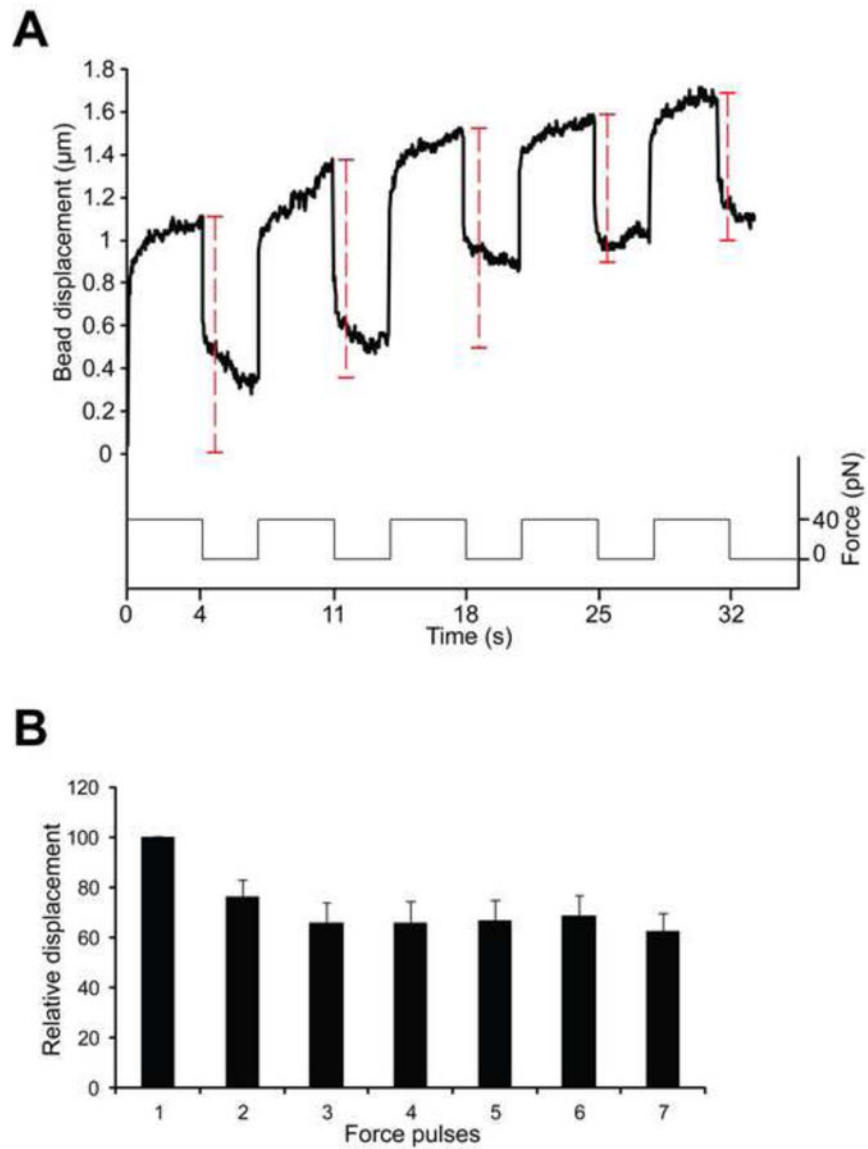


Figure 4. Cellular stiffening of HUVECs in response to pulses of force applied to hVEC-Fc magnetic beads using magnetic tweezers. **A**, Typical displacement of a 2.8 µm hVEC-Fc bead bound to HUVEC during application of cycles of 4 sec extension (force on: 40 pN) and 3 sec recovery (force off). Decreasing displacement is highlighted in red, demonstrating cellular stiffening. **B**, Change in bead displacement during 7 force pulses applied to hVEC-Fc beads (n=14). Displacements were calculated relative to the displacement generated by the first pulse of force (error bars represent SEM).