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Heterogeneous elastic response of human lung microvascular endothelial cells to barrier modulating stimuli

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Abstract

In this study we employ atomic force microscopy, supported by finite element analysis and fluorescence microscopy, to characterize the elastic properties accompanying cytoskeletal structural rearrangements of lung microvascular endothelial cells in response to barrier altering stimuli. Statistical analysis of elasticity data obtained from multiple cells demonstrates a heterogeneous cellular elastic response to barrier-enhancing and barrier-disrupting agents; sphingosine 1-phosphate (S1P) and thrombin, respectively. A small, but detectable (10%) increase in the average elastic modulus of all cells is observed for S1P, which is accompanied by a corresponding significant decrease in cell thickness. Variable effects of thrombin on these parameters were observed. To account for possible substrate effects in our elasticity analysis, we analyzed only the low-force sections of the force-displacement curves and utilized a finite-thickness correction to the Hertzian model. Our finite element analysis results substantiate this approach. The heterogeneous elastic behavior correlates with differential cytoskeletal rearrangements observed with fluorescence microscopy.

Keywords

F-actin; cell; AFM; FEM; elasticity

BACKGROUND

The endothelial cell (EC) layer of the pulmonary vascular system forms a semipermeable barrier between the blood and pulmonary interstitium.¹ Disruption of this barrier occurs in multiple inflammatory disease processes, resulting in increased permeability of fluid and macromolecules into the interstitium and air sacs of the lung,^{1, 2} often leading to pulmonary edema and respiratory failure. Barrier enhancing agents, such as sphingosine 1-phosphate (S1P), are the subject of intense study because of their ability to decrease vascular

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permeability and increase barrier integrity by strengthening intercellular and cell-matrix adherence.³⁻⁵

Actin filaments form a dynamic network in the EC cytoskeleton, being able to undergo structural rearrangements as a result of external stimuli such as barrier modulating agents. In the pulmonary endothelium, actin acts as an essential regulator of endothelial permeability and is closely linked to EC barrier modulation. Agonist-induced rearrangement of actin filaments results in changes of cell shape and altered cell-cell and cell-matrix linkages combining to modulate EC barrier function.^{1, 3-5} Recent work suggests that these structural changes are associated with changes in the elastic modulus of ECs.⁶⁻¹⁰

Elasticity in cells plays a fundamental role in adapting the cellular shape to different environmental conditions, as well as during cell migration and division. Even though the elastic modulus of different cell types has been studied using atomic force microscopy (AFM) and other techniques,¹¹⁻¹⁴ accurately determining cell elasticity remains a challenging problem due to the extreme softness of cells, the reduced number of experimental techniques available and difficulty in obtaining statistically significant data due to the significant biological variability involved in the differential responses of cells. During AFM exploration of cellular elastic properties, a set of force-displacement curves is acquired by indenting the AFM tip at various locations of the cell, and the elastic modulus is obtained by fitting the resulting indentation curves with an appropriate theoretical model. Most commonly used is the Hertz model,¹⁵ which assumes a linearly elastically deformable medium of infinite thickness indented by a sphere. A more accurate analysis requires finite thickness corrections to the Hertz model, e.g. using the Dimitriadis correction,¹⁶⁻¹⁹ in particular at the thinner regions of the cell periphery.

We previously used AFM to characterize changes in the elastic modulus of human lung ECs in response to actin rearrangement in the cytoskeleton caused by barrier modulating agents.⁶ These prior experiments were limited by use of fixed EC, adding the artifact of protein crosslinking and complicating the detection of small changes in elasticity of the cells. In the current study, we perform AFM analyses in living cells by investigating the effects of two barrier modulating agents, barrier enhancing S1P and barrier disrupting thrombin, on the elastic modulus of live human lung micro vascular (HMLV) EC. Our results reveal a small increase in the elastic modulus, averaged over all cells stimulated in our study. This correlates with the peripheral rearrangement of actin observed with fluorescence microscopy.

MATERIALS AND METHODS

Reagents

Reagents (including S1P and thrombin) were obtained from Sigma (St. Louis, MO) unless otherwise specified. Texas Red phalloidin was purchased from Invitrogen (Carlsbad, CA).

Cell culture

Human lung microvascular endothelial cells (HMLVEC) obtained from Lonza (Walkersville, MD) were cultured in the manufacturer's recommended EBM-2 complete medium at 37°C in a humidified atmosphere of 5% CO₂/95% air, with passages 5-9 used for experimentation.

Immunofluorescent imaging

EC were grown on gelatinized coverslips before exposure to various conditions as described for individual experiments. EC were then fixed in 3.7% formaldehyde for 15 min, permeabilized with 0.25% Triton X-100 for 5 min, washed in PBS (phosphate buffer saline),

blocked with 2% BSA (bovine serum albumin) in TBST (tris buffer saline with Tween 20) for 1 hr, and then incubated for 1 hr at room temperature with Texas Red-conjugated phalloidin for actin staining. After washing with TBST, coverslips were mounted using SlowFade® Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole from Invitrogen, Grand Island, NY) and analyzed using a Nikon Eclipse TE 300 microscope and Sony Digital Photo camera DKC 5000. Images were recorded and saved in Adobe Photoshop.

Finite Element Analysis

The mechanical properties of the cells were simulated using a finite element model (FEM) with Abaqus 6.10 (Providence, RI). The indentation of adherent cells by an AFM cantilever with a spherical bead attached to the end was modeled as axisymmetric compression of linear-elastic films with a rigid spherical probe. Due to the high stiffness of the silicon bead (170 GPa) when compared to the stiffness of the cells (0.5-5 kPa), the probe was assumed to be rigid. The indenter was modeled as a sphere with 2.5 μm radius (R), consistent with the dimensions used in the experiments. The elastic properties of cell regions with thickness (H) were modeled using thin slabs of 10 μm radius (r) (Figure S1), selected to be large enough so that the stress transfer would be insignificant at the edges. The local thickness of the cell was varied in the range of 0.2-5 μm , which reflected the values observed in the AFM data. In the indentation experiments, the cells were compressed up to a value of 2 nN. This was modeled by applying a concentrated load ramped linearly from 0 to 2 nN on the indenter in the direction parallel to the axis of symmetry. Four node axisymmetric elements with mesh biasing near the point of contact were used to mesh the cell with a total number of 9100-10500 elements depending on the model cell thickness (Figure S1). Convergence of results was confirmed through mesh refinement studies.

The boundary conditions for the model were defined in the following manner. 1) All nodes on the bottom edge were fixed, assuming the cells are firmly adhered to the plate. 2) Due to symmetry constraints, nodes lying on the axis of symmetry were only allowed motion parallel to the axis of symmetry 3) Indenter motion was restricted to the axis of symmetry with no rotation. Contact between the indenter and the cell was assumed to be frictionless. From each simulation, force-displacement curves were extracted. The force-displacement curves were fit to the Dimitriadis and Hertz models for indentation.^{15, 16, 20} The elastic modulus from the force-displacement curve was approximated through non-linear curve fitting with Matlab 7.10 (MathWorks, Natick, MA). In the AFM experiments, only forces up to 0.2 nN were used to determine the mechanical properties of the cell. In order to validate this experimental procedure, Hertz and Dimitriadis models were fit for the whole force-displacement data collected with FEM (up to 2 nN) and for the partial curve (up to 0.2 nN).

AFM data acquisition and analysis

All measurements were carried out with a Bioscope AFM (a prototype of Digital Instruments Bioscope) from Bruker (Santa Barbara, CA) integrated with a Zeiss Axiovert 135TV (Carl Zeiss, Germany) inverted light microscope. The AFM was equipped with a 150×150 μm^2 ("G") scanner and run by the 5.31R1 Nanoscope software from Bruker. Mechanical measurements of live cultured cells were performed at room temperature in contact mode inside plastic Petri dishes 3.5 cm in diameter filled with ~ 2 mL cell media. To reduce stress in cells during indentation experiments, borosilicate glass beads 5 μm in diameter attached to tipless Si_3N_4 cantilevers (Novascan Technologies, IA) with nominal spring constants of 0.06 N/m were employed. Measurements were carried out by acquiring arrays of 32×32 force vs. distance curves (force-volume) as described elsewhere.^{6, 21} A maximum load of ~ 3 nN was applied at each data point. The tip velocities for these measurements varied usually between 6 $\mu\text{m/s}$ and 10 $\mu\text{m/s}$. The sensitivity of the

photodetector was calibrated by acquiring force vs. distance curves on a clean glass substrate. During a typical experiment, a large scale AFM image was rapidly acquired at a relative low number of pixels, typically 512×64 , to locate an individual cell appropriate for measurements. Elasticity data on this cell were acquired in 30×30 - $35 \times 35 \mu\text{m}^2$ areas over time periods of ~ 30 min before S1P or thrombin stimulation. Cells were stimulated by adding 10-20 μL solutions of S1P or thrombin to the Petri dish to reach a final concentration of 1 μM (S1P) or 1 unit/mL (thrombin) inside the dish during data acquisition.⁶ After agonist stimulation, elasticity measurements were continued for ~ 1 hr in the same region of the cell.

A custom MatLab code, described in detail elsewhere,^{6, 21} was developed for statistical analysis and to obtain elasticity maps from the force volume raw data. Briefly, our program allowed data points to be selected either manually or by choice of height intervals in the height image from the force volume file. The force curves in these regions of the cell were subsequently transformed into indentation curves and fitted according to the Hertzian and Dimitriadis models to obtain the elastic moduli^{15, 16, 20}. Only curve regions with loads up to 0.2 nN were fitted to ensure linear deformations and justify the use of the Hertz or Dimitriadis models. The local thickness of cells at each data point was determined by adding the cell deformation, obtained from the indentation curve, to the height value obtained from the height image in the force volume file. This was the value used to correct for finite thickness in the Dimitriadis model.²²

When the local thickness was considered too low (< 200 nm) or the elastic modulus obtained from the Hertz model exceeded the values found typically in the analysis (3-5 kPa), the data point was excluded from further analysis and not included in the statistics. The same procedure was followed for data points that exceeded typical values for the elastic modulus, regardless of the specimen's thickness in the region. Cell regions with local thicknesses close to the maximum vertical range of the scanner ($\sim 5 \mu\text{m}$) produced force curves plagued with artifacts when data points of the curve corresponded to vertical positions (Z-positions) beyond the scanner's range. The force curves in those regions had deflections identical to zero, when the Z-position was outside the scanner's range, that could abruptly increase to larger values once the Z-position came within the scanner's range with the cantilever in contact with the cell. Therefore, a maximum thickness threshold was set to exclude these curves from further analysis.

RESULTS

Stress analysis in cells

To begin to characterize the elastic properties of live barrier-regulatory HMVEC, FEM of cell indentations was performed to validate the procedures used in the AFM analysis. In order to achieve this, axisymmetric compression of a slab with finite thickness was utilized to simulate the behavior of the cells. Fig 1 shows the Mises stress (defined as $1/3$ the sum of the principal stresses along the diagonal of the stress tensor) in the FEM of a $0.5 \mu\text{m}$ thick cell model ($E = 1$ kPa) under compression by a rigid spherical indenter. When high forces (~ 2 nN) are applied to the slab (Figure 1, A), elevated stresses are present at the top surface and are transferred across the thickness of the sample to the base through the action of the rigid indenter and rigid base. With lower forces (~ 0.2 nN), low basal stresses still subsist due to the presence of the hard substrate and the low elastic modulus of typical cells (< 5 kPa) (Figure 1, B). Models with thicknesses exceeding $3 \mu\text{m}$ and low elastic modulus ($E=0.5$ kPa) do not show elevated basal stresses for forces up to 0.2 nN. Similar results were obtained for models with 1.5 μm thickness and larger elastic modulus (5 kPa). Therefore, it is necessary that forces of 0.2 nN or less be used to reduce substantial effects of the substrate on the values found for the elastic modulus of cells.

Indentation curves were generated for multiple thicknesses and stiffnesses with FEM to determine whether the models agreed with the AFM results. This provided insight into the range of forces and deformations in which the experimental behavior agrees with the linear assumptions used in the FEM models. When compared to experimental data, the FEM curves demonstrated agreement with experimental curves in the low force regime (<0.3 nN) even for small thicknesses (~ 0.5 μm) (Figure 2, A). However, for these thicknesses, the FEM generated curves started to deviate from the experimental data at higher forces. In the example shown in Figure 2, A, the AFM data agree closely with the FEM models when the cell is compressed approximately 20% of its total thickness for $E = 1.23$ kPa and a matched thickness of 0.45 μm obtained from the AFM data. Thus, the models agree well with the AFM data in the low force regime for the typical range of cell thicknesses (0.2 to 3 μm) observed in the data. This validates that lower forces result in behavior similar to linear models and forces of 0.2 nN result in curves with high similarity between experimental and theoretical FEM behavior.

EC stimulation with barrier enhancing agent, S1P

To study differential effects of cytoskeletal reorganization on the elasticity and morphology of live pulmonary EC, AFM measurements on the same individual cell were carried out before and after cell stimulation with the barrier modulating agents, S1P and thrombin. Data collected at the cell periphery and along the entire cell were analyzed to correlate these elasticity measurements with peripheral cytoskeletal actin rearrangement observed using optical fluorescence microscopy.⁶ Two measurements performed on each cell before stimulation served as internal controls. An example of serial measurements in the same individual cell before and after S1P stimulation is shown in Figure 3. The summarized results for all cells are shown in Figure 4 (AFM results for S1P), Figure 5 (fluorescence results for S1P and thrombin) and Figure 6 (AFM results for thrombin). The bars in Figure 4, A, B, E and Figure 6, A, B, E represent results for each individual cell before and after stimulation (each color specifies the results for a single cell serially evaluated over time). The plots in Figure 4, C, D and Figure 6, C, D display average values over the cells. In these plots, cell thickness and elastic modulus values are normalized with respect to their initial values, i.e. the second measurement before cell stimulation with S1P or thrombin. Therefore, the normalized elastic moduli (E/E_0) do not depend on the specific value of the spring constant of the cantilever used. E_p/E_N is the ratio of the elastic modulus obtained at the cell periphery (dotted regions in Figure 3 and bars in Figure 4, B and Figure 6, B) and on the nucleus (marked by crosses in Figure 3).

For the potent barrier enhancing agent S1P, reorganization of the cytoskeleton at the periphery of the cell resulted in a ~ 10 % decrease in the maximum cell thickness, H , over the course of the experiment (~ 50 min) (Figure 3 and Figure 4, C). Since the cell deformation induced by the AFM probe was added to the raw height (Figure 3, A) to produce the local thickness (Figure 3, B), the local thicknesses are larger than the raw heights obtained directly from the AFM Nanoscope software. Even though cytoskeletal reorganization is observed in both of these images (Figure 3, A and Figure 3, B), the morphological details revealed by these analyses at the cell periphery are somewhat different.

Values in the order of ~ 1 kPa were generally measured for the elastic modulus (E) of the examined cells after taking into account the finite thickness correction of the Dimitriadis model (Figure 3, C, D, E).¹⁶ As explained with our FEM cell models (Figure 2, C), larger absolute values were obtained when the Hertz model was used to fit the experimental data (Figure 3, E). Elastic modulus values obtained from both models are within the range of those reported in other AFM studies of live EC (1.3 - 14 kPa)^{8, 10, 23-25}. Both Dimitriadis and Hertz models resulted in relative increases in EC elastic modulus values, thus indicating an

increase in cytoskeletal rigidity as a result of S1P stimulation. Elasticity maps (Figure 3, D and Figure 3, E) demonstrate increased values of elastic modulus somewhat localized in the peripheral cell region.

Following S1P stimulation, histograms of the elastic moduli in the cytoplasmic region (i.e., excluding values over the nucleus) (Figure 3, C) showed an increase in the number of the larger values ($E > 1.5$ kPa), as well as a decrease in the number of lower values. This resulted in a 15-20% increase of the average elastic modulus over the cytoplasmic region of this cell, which represents one of the largest increases detected for all the cells studied. It is important to note that only data corresponding to cell thicknesses > 250 nm and cell deformations less than 20% of the local thickness were included in the analysis to avoid results to be influenced by the rigidity of the Petri dish substrate. As a result, our data likely represent an underestimate of potential elastic modulus changes at the periphery where cell thickness can be < 200 nm. Nevertheless, we believe that these data provide a relevant lower estimate for dynamic agonist-mediated elasticity changes relevant to endothelial function in live HMVEC. The changes in elastic modulus evaluated for the entire cell largely mimicked the results at the cell periphery where measurements were available within the thickness parameters outlined above. The central region of the cell, corresponding to the cell nucleus, was thicker and therefore more susceptible to errors arising from saturation of the vertical scanner displacement (~ 5 μ m). Therefore, due to the lower number of significant data points in this region, its elastic modulus, E_N , was analyzed for only a subset of cells (6 out of 9).

Immunofluorescent analysis of HMVEC actin structure and morphology reveals a differential response to S1P stimulation (Figure 5). Before stimulation, HMVEC exhibit a cobblestone morphology with F-actin primarily distributed at the cell periphery with few intercellular gaps (Figure 5, A). After S1P (1 μ M, 20 min) (Figure 5, B), a broader range of cytoskeletal and morphological patterns are observed, with some cells demonstrating increased cortical actin as previously described,^{3, 6, 28} others exhibiting increased actin stress fibers similar to those produced by thrombin,^{1, 6, 29} and some that appear relatively unchanged with characteristics most resembling vehicle-treated EC.

EC stimulation with barrier disrupting agent, thrombin

While a small, but detectable increase in elastic modulus was observed at the cell periphery following stimulation with the barrier enhancing agent S1P, no significant change was seen with the barrier disrupting agent thrombin (Figure 6). The average height increased less than 5% after 50 min thrombin stimulation, and the average elastic modulus underwent a 5% increase in that time, but neither of these parameters was significantly changed from baseline values. There is a modest increase ($\sim 20\%$) in the $\langle E_P/E_N \rangle$ ratio, ~ 35 minutes upon stimulation with thrombin (Figure 6, D). This result, however, is smaller than what was observed for S1P and it also has a larger error. Notably, similar to S1P-treated cells, there is a 19% decrease before thrombin stimulation. Furthermore, similar to the results for S1P-treated cells, the average elastic modulus at the periphery of cells is greater than at the central region at all time points, but there is considerable variability among individual cells (Figure 6, E). Data for E_N at -20 min, the first measurement before thrombin stimulation was acquired for only 3 cells.

Substantial heterogeneity is observed in HMVEC morphology and F-actin structure after thrombin (Figure 5). Although most cells exhibit the increased actin stress fiber pattern typical of thrombin stimulation (1 unit/ml, 20 min), a substantial fraction appear relatively unchanged from baseline, while others demonstrate increased cortical actin similar to post-S1P cells. While only a subset of HMVEC stimulated with thrombin undergo the morphological changes and F-actin redistribution leading to increased stress fiber formation,

most cells appear smaller and more rounded than vehicle treated EC, and large intercellular gaps are present (Figure 5).

DISCUSSION

Thickness greatly affects the stress distribution along the surface of the cell. Indenting cells the same amount results in varying radial stress distributions around the center of the indenter, with dependence on the force and geometry. Cell models with thicknesses of 0.5 μm and 3 μm , $E=1$ kPa, compressed at 2 nN demonstrate elevated stresses at the top surface, with the stresses nearly twice as large in the 0.5 μm model (Figure 1, C). Independent of slab thickness, basal stresses were present in all cell models, but they were much higher for the thin models at forces of 2 nN (Figure 1, C). In spite of the high stresses seen in the models, there is a sharp decrease in the stress as a function of distance from the axis of symmetry of the indenter (Figure 1, A, B, C). This indicates that stress transfer is minimal over long radial distances and suggests that the mechanical properties measured are governed by the local properties under the indenter allowing for local mechanical properties to be measured with our experimental protocol. While at the top of the cell the indentation produces the highest stress in regions directly under the indenter (Figure 1, C), the maximum basal stress occurs a few μm from the symmetry axis (Figure 1, C) due to stress transfer from the substrate.

Though the Hertzian model appears to agree well with the AFM data (Figure 2, B), it continually over-estimates the elastic modulus of the cells while the Dimitriadis correctly estimates the value in the low force (< 200 pN) regime of indentation (Figure 2, C). At higher indentation depths, the elastic modulus is highly overestimated by the Hertzian model and is underestimated by the Dimitriadis due to the effects of the stiff substrate (Figure 2, C). The same result was seen for elastic moduli between 0.5-5 kPa, values typical of cell mechanics. The Dimitriadis model demonstrates accurate approximation of the local mechanical properties of thin cells when small forces are applied. This model correctly estimates the FEM mechanical properties which also show strong correlation to the AFM models at low forces with the same stiffness.

Out of 9 different cells examined before and after being stimulated with S1P, 8 of them consistently decreased their height in the first 17 minutes following stimulation (Figure 4, A), and all cells, on average, significantly decreased their maximum height by approximately 10% after 50 minutes (Figure 4, C). Our results indicate that S1P alters pulmonary EC structure to assume a flatter conformation, possibly leading to more cell-cell overlap and reduced permeability. However, S1P effects on the elastic properties of live pulmonary EC were less uniform. An increase in elastic modulus was measured for 5 out of 9 of these cells (Figure 4, B), which in turn produced a 9% increase in the overall average elastic modulus (Figure 4, C). Although this increase failed to reach statistical significance, our current results follow the trend that we previously observed in live HMVEC after S1P treatment for a reduced sampling of cells ($N=4$).²⁶ Possible reasons for the difference in observed increases of E/E_0 ($\sim 30\%$ in our previous study vs. 9% in our current data) include the biological variability inherent in differing cell lots, the heterogeneity of cellular responses within an individual EC monolayer, as well as a possible overestimation of elastic moduli due to inclusion of larger stresses and exclusion of the finite thickness corrections from the Dimitriadis model in our previous data analysis. These last two effects are likely to alter the analysis after S1P stimulation due to the decrease in cell thickness, thus possibly contributing to the larger E/E_0 increase noted in our previous study. Nevertheless, in the current study, a steady increase in the average ratio of the peripheral elastic modulus with respect to the central elastic modulus, $\langle E_P/E_N \rangle$, (Figure 4, D) was observed upon stimulation with S1P. This result, although not statistically significant, follows the same

trend observed in our previous study utilizing fixed cells.⁶ The highest increase (~ 35%) occurs after ~ 50 minutes of S1P stimulation. A sharp decrease (~ 25%) occurs between measurements before S1P stimulation. We speculate that this is due to cell perturbation produced by the cantilever during the measurement. Thus the steady increase observed subsequently might signify cytoskeletal recovery produced by the action of S1P. On average, the values of elastic modulus at the periphery of cells are larger than on their central region ($E_p/E_N > 1$). However, there is considerable variability among single cells, especially after thrombin as displayed in Figure 6, E (see below). Although only 2 cells have $E_p > E_N$, before S1P stimulation, E_p becomes larger than E_N for 5 cells ~ 55 minutes after stimulation.

Recent work has identified significant variations in mechanical stress exhibited by individual cells in migrating endothelial monolayers²⁷. Similarly, in our experiments it is likely that subpopulations of HMVEC respond to S1P stimulation with differential cytoskeletal and biomechanical effects, with some EC exhibiting increased elastic modulus values and others with little change or even decreased values (Figure 4, B). Support for this hypothesis is provided by immunofluorescent analysis of HMVEC actin structure and morphology before and after S1P stimulation (Figure 5). S1P (Figure 5, B) induces a broad range of cytoskeletal and morphological patterns, with some cells demonstrating increased cortical actin as previously described,^{3, 6, 28} others exhibiting increased actin stress fibers similar to those produced by thrombin,^{1, 6, 29} and others that are relatively unchanged from baseline. These differential cytoskeletal patterns are likely to produce heterogeneous elastic modulus values after S1P as detected in our measurements (Figure 4b and 4c). Substantial variability is observed from experiment to experiment, but in general ~50-75% of HMVEC exhibit the increased cortical actin pattern associated with S1P barrier enhancement (data not shown). Thus, the increase in elastic modulus detected here in 55% of the cells examined appears to correlate with these observations. Taken together, our results suggest that rather modest increases in cytoplasmic elastic modulus values of only a fraction of cells may be sufficient to produce the barrier-strengthening physiological effects observed in pulmonary endothelium after S1P. Trans endothelial resistance (TER) measurements demonstrate increased pulmonary EC TER values following S1P exposure,^{3, 6, 28} thus correlating the mechanical increase in elastic modulus and morphological spreading of cells with a functional decrease in barrier permeability and enhanced endothelial junctional integrity of the EC layer.

Multiple previous TER and optical immunofluorescence microscopy studies have demonstrated that thrombin induces morphological transcellular stress fiber formation and F-actin redistribution associated with intercellular gap formation, functionally leading to increased permeability in cultured EC.^{1, 6, 29} Therefore, a seeming increase in average cell height and elastic modulus would have been expected from those results, but this was not found in our current AFM measurements (Figure 6). This surprising result, again, may be due in part to the substantial heterogeneity observed in HMVEC morphology and F-actin structure after thrombin (Figure 5). While most cells exhibit the increased actin stress fiber pattern typical of thrombin stimulation,^{1, 6, 29} many appear relatively unchanged from baseline or demonstrate increased cortical actin similar to post-S1P cells. However, most cells appear smaller and more rounded than vehicle treated EC, and large intercellular gaps are present (Figure 5). Thus, although induction by thrombin of the contractile phenotype in only a subset of HMVEC appears sufficient to increase EC permeability, these changes may not be sufficient to produce significant alterations in the average height and elastic modulus across all cells. From our results (Figure 6), only ~ 45% of all cells treated with thrombin increased their height (and 33% increased their peripheral elastic modulus) 17 minutes after stimulation, and this percentage increased to 55% after 50 min, which produced the mild increases observed in Figure 6, C.

In summary, in the current study, we have studied the elastic response of live HMVEC to barrier-enhancing (S1P) and barrier-disrupting (thrombin) stimuli using elasticity-mapping with AFM, as well as FEM modeling and fluorescence microscopy. Our combined results demonstrate the substantial heterogeneity in individual cellular responses to these agonists despite the highly reproducible and potent effects on permeability. A decrease in the average cell thickness, accompanied by an increase in the average elastic modulus at the cellular periphery, was observed upon S1P stimulation. Further investigation is warranted into how these varied individual cell responses are determined and integrated into physiologically relevant EC monolayer system responses. Our results also provide quantitative parameters for the necessity of finite thickness corrections to the Hertzian model for accurate analysis of elasticity data from AFM indentations on the thinner cellular regions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FEM results demonstrating the stress distribution in model cells

Cell regions with varying thicknesses were modeled as thin slabs of uniform elastic modulus located on a rigid substrate for indentations by a sphere with 2.5 μm radius (Figure S1). **(A)** When a 2 nN force is applied on a 0.5 μm thick cell region, elevated stresses exist at the point of indentation and at the base. **(B)** Conversely, for a 0.2 nN load applied on the 0.5 μm cell region, the basal stresses are lower, thus indicating less substrate effects on the stress distribution and approaching the behavior of a medium with infinite thickness described by the Hertzian model. **(C)** Stresses at the top (in black lines) and basal (in red lines) surfaces plotted as a function of distance from the axis of symmetry for 0.5 μm (solid lines) and 3 μm (dashed lines) thick cell regions, with $E=1$ kPa, under an applied force of 2 nN. Plots show elevated stresses at the top and smaller at the base.

Figure 2. Force-displacement data generated from FEM compared to Hertzian and Dimitriadis linear theoretical models and experimental AFM data

(A) FEM force-displacement curves for a 2 nN maximum load indentation on cell regions with 0.45 μm and 2 μm thickness and $E = 1.23 \text{ kPa}$. FEM results are compared to AFM data for samples of the same thickness and elastic modulus. E was determined from these data using the Dimitriadis model. (B) A force-displacement curve for 10% compression of a 0.45 μm thick sample is compared across models. Dimitriadis and Hertzian fits are shown along with a portion of the AFM data used in (A). (C) Elastic moduli obtained from the Hertzian and Dimitriadis models are compared to FEM data as a function of local cell strain. The value $E = 1 \text{ kPa}$ obtained from FEM data is independent of strain and represents the expected behavior. The curves are representative of cell regions with thicknesses within a range of 0.2–5 μm . The Dimitriadis model demonstrates a very good approximation to the expected elastic modulus at low forces (0.2 nN) and 20% strain used in our AFM analysis. Even for a 2 nN force and larger strains, it approximates the expected result more closely than the Hertzian model. All fits had R^2 values exceeding 0.95, but the Hertzian model still overestimated the elastic modulus. Data were fit for maximum indentation forces of 2 nN and 0.2 nN.

Figure 3. Correlation of cytoskeletal morphology and elasticity at different time points following stimulation with S1P

(A) Shown are local height values obtained directly from the AFM height image. Each figure represents the same cell analyzed over time. $t = 0$ corresponds to the time point when the last measurement was completed before cell stimulation with S1P ($1 \mu\text{M}$). Other times listed reflect either pre or post S1P stimulation. (B) Local thickness, obtained by adding the cell deformation to the local height, is shown for the same cell at each condition as in (A) above. (C) Histograms of elastic moduli for the cytoplasmic region obtained by using the Hertzian and Dimitriadis models to fit 0.2 nN sections of the indentation curves. Gray and white bars represent results from the Dimitriadis and Hertzian models, respectively. (D) Maps of elastic moduli obtained from the Dimitriadis model and (E) the Hertzian model. The color scale is the same for both maps. Increased values at the periphery of the cell are seen for the Hertzian model. Regions void of dots or crosses were deemed too thin ($< 250 \text{ nm}$), deformations too large compared to cell thickness ($> 20 \%$) or produced elastic moduli

outside a reliability interval set to exclude outliers. These regions were excluded from statistical analysis. Regions marked with crosses were associated with the nucleus and were also excluded from cytoplasmic elasticity analysis. Mean elastic moduli values are listed above each map.

Figure 4. Changes in maximum cell height and elastic modulus as a function of time following SIP stimulation

(A) Changes over time in the maximum cell height, H , normalized with respect to its initial value, H_0 . Each bar represents measurements taken from the same individual cell over time. A total of 9 cells were analyzed. (B) Corresponding changes over time in the average cytoplasmic elastic modulus, E , normalized with respect to the value at the initial time point, E_0 . Bars represent measurements taken for the same individual cell over time. (C) The average values of H/H_0 and E/E_0 for all cells demonstrate a trend toward small, progressive increases in the elastic modulus associated with steady decreases in cytoplasmic thickness after SIP stimulation. The maximum cell height was found to be significantly different from the initial value according to a student's test performed at the $p^*=0.05$ level. The increased trend in the elastic modulus did not reach statistical significance. $N = 9$ cells analyzed in multiple independent experiments. (D) Ratio of elastic moduli at the periphery, E_p , and the central region, E_n , averaged for 6 of the cells analyzed in (A, B). An insufficient number of data points in the central region precluded the determination of this value for the remaining cells. (E) Ratio of elastic moduli at the periphery and the central region for the individual cells analyzed. The bar colors used to represent the individual cells are the same as those used in (A, B).

Figure 5. Heterogeneity of HMVEC F-actin distribution after S1P and thrombin

HMVEC were plated as described in Methods and then stimulated with (A) vehicle control, (B) S1P (1 μ M), or (C) thrombin (1 unit/ml) for 20 minutes. After fixation, Texas-red phalloidin was used to characterize the F-actin structure. Compared to vehicle, S1P stimulation produced increased cortical actin (arrows) in some cells and increased stress fibers (arrowheads) in others. Thrombin induced large intercellular gaps (asterisks), increased stress fibers (arrowheads) in some cells, and increased cortical actin (arrows) in others. Representative images are shown from multiple independent experiments. Scale white bar = 10 μ m.

Figure 6. Maximum cell height and cytoplasmic elastic modulus as a function of time following thrombin stimulation

Absence of values for 2 cells at $t=-20$ min is due to the lack of measurements at this time point. **(A)** Changes in maximum cell height over time after thrombin stimulation (1 unit/ml), compared to measurements before stimulation. Each bar represents measurements taken from an individual cell over time. **(B)** Corresponding changes over time in the elastic modulus, E/E_0 , normalized with respect to the initial value. **(C)** Average values of H/H_0 and E/E_0 for all cells. A student's t-test at the $p^*=0.05$ level found that the population means of the normalized elastic moduli and heights after thrombin were not significantly different from baseline. $N = 9$ cells analyzed in multiple independent experiments. **(D)** Ratio of elastic moduli at the periphery, and the central region, E_p/E_N , averaged for 6 cells from (A) and (B). **(E)** Results of E_p/E_N for the individual cells. The bar colors follow those used to represent the individual cells in (A, B).