Chapter 3

Traction force dynamics predict gap formation in activated endothelium

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Abstract

In many pathological conditions the endothelium becomes activated and dysfunctional, resulting in hyperpermeability and plasma leakage. No specific therapies are available yet to control endothelial barrier function, which is regulated by inter-endothelial junctions and the generation of actomyosin-based contractile forces in the context of cell-cell and cell-matrix interactions. However, the spatiotemporal distribution and stimulus-induced reorganization of these integral forces remain largely unknown.

Traction force microscopy of human endothelial monolayers was used to visualize contractile forces in resting cells and during thrombin-induced hyperpermeability. Simultaneously, information about endothelial monolayer integrity, adherens junctions and cytoskeletal proteins (F-actin) were captured. This revealed a heterogeneous distribution of traction forces, with nuclear areas showing lower and cell-cell junctions higher traction forces than the whole-monolayer average. Moreover, junctional forces were asymmetrically distributed among neighboring cells. Force vector orientation analysis showed a good correlation with the alignment of F-actin and revealed contractile forces in newly formed filopodia and lamellipodia-like protrusions within the monolayer. Finally, unstable areas, showing high force fluctuations within the monolayer were prone to form inter-endothelial gaps upon stimulation with thrombin.

To conclude, contractile traction forces are heterogeneously distributed within endothelial monolayers and force instability, rather than force magnitude, predicts the stimulus-induced formation of intercellular gaps.
Introduction
The vascular endothelium forms a physical, dynamic barrier between the blood and the surrounding tissues, actively controlling the passage of fluids, proteins and cells. In inflammatory conditions, endothelial barrier function is disturbed leading to vascular leakage and impaired organ function.1 Hyperpermeability of the endothelial monolayer is a hallmark of many life-threatening inflammatory diseases and despite its medical importance, no specific therapies are available.

Contractile forces play an important role in the regulation of endothelial barrier integrity.2–6 Vasoactive agents like thrombin increase intracellular Ca2+ levels,2,7 and phosphorylation of myosin light chain and junctional proteins.4,8,9 For thrombin and VEGF, this is accompanied by rapid activation of the GTPase RhoA.10,11 Rho family GTPases control the actin cytoskeleton and its associated myosin II motor activity and are key regulators of permeability. RhoA-driven formation of filamentous actin (F-actin) stress fibers and actin-myosin interaction generates tension which is transmitted to F-actin-linked proteins at cell-cell junctions, leading to disruption of inter-endothelial connections.1,11,12 In endothelial cells, force-dependent mechanosignaling takes place at cell-cell junctions through vascular endothelial cadherin (VE-cadherin),13–15 which regulates vascular barrier function in vitro and in vivo16–18 and through focal adhesions, which, via integrin-F-actin complexes, exert traction forces on the extracellular matrix.19–21 Focal adhesions mature from unstable, smaller focal complexes by force-induced recruitment of integrins, F-actin and linker proteins.22,23 As a consequence, cell-matrix interactions are reinforced and traction forces increase. Traction force microscopy allows quantification of such forces via the displacement of fiducial markers crosslinked to the surface of a deformable matrix.24 Previous studies showed that traction forces regulate endothelial cell sprouting,25 spreading26 and migration27 and respond to mechanical cues such as fluid shear stress,28,29 substrate stiffness30 and stretch.31 Moreover, traction forces are induced by age-associated changes32 and vaso-active agents.33,34 However, the spatiotemporal distribution of traction forces in resting and activated endothelial monolayers remains largely unknown.

Here, we investigated the localization and dynamics of traction forces within confluent endothelial monolayers to obtain new insights into the mechanisms that drive vascular leakage. Analysis of junctional- and nuclear areas revealed heterogeneous force distribution within endothelial cells. Thrombin induced a rapid increase in traction forces, formation of inter-endothelial gaps and the generation of opposing contractile forces at gap boundaries. Finally, we show that intercellular gaps were prone to form at monolayer regions marked by force fluctuations, rather than force magnitude.
Material and Methods

Cell culture and preparation of polyacrylamide gel substrates
Sources of reagents are listed in the expanded Materials and Methods section in the online data supplement section. Umbilical cords of healthy donors were provided by the Department of Obstetrics from the Amstelland Hospital (Amstelveen, The Netherlands) in accordance with the principles outlined in the Declaration of Helsinki. Isolation of human umbilical vein endothelial cells (HUVECs) was achieved as described previously. Polyacrylamide hydrogels with collagen ligation and embedded fiducial markers were produced as previously described. Subsequently, a concentrated cell suspension of HUVECs was directly added to the center of each gel and grown for two consecutive days to confluence.

Traction force microscopy
Before the start of the experiment, the adherens junctions (VE-cadherin) or the F-actin cytoskeleton of the confluent HUVEC monolayers were stained. The cells were visualized using a Zeiss Axiovert 200 MarianasTM wide-field inverted microscope that was equipped with a climate-controlled universal heating- and gas incubation system (Ibidi, Planegg, Germany; temperature: 37°C, CO2: 5%, humidity: 80%). Cell morphology was imaged in differential interference contrast (DIC), and the VE-cadherin or F-actin, in combination with top- and reference beads for the computation of traction forces were imaged using fluorescence. After a baseline period, the HUVECs were stimulated with 1 U/ml of the vaso-active agent thrombin. The last step consisted of the trypsinization of the endothelial monolayer from the substrate to acquire an unloaded fiducial marker pattern, after which a final image of all positions was captured. In order to determine monolayer traction forces, we used the well-established constrained two-dimensional fast Fourier transformation method, with the prior knowledge of the substrate material properties (substrate stiffness = 1.2 kPa, Poisson’s ratio=0.48). From the monolayer traction fields, we calculated the root mean squared (RMS) value of traction in Pascal, which is a scalar measure of the cell’s net contractile strength.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 5 (GraphPad Soft-ware, San Diego, CA, USA). Statistical significance of the data represented in Figure 2A was tested using a Spearman’s rank correlation, whereas a Mann Whitney test and the McNemar test were conducted for 5A and 6B, respectively. Moreover, a student t-test was used on the data of Figure –II and a repeated measures ANOVA with Bonferroni post-hoc test was applied on the data shown in Figure 4 and SIII. The numbers of replicates and significant p-values are indicated in the text of each figure. Results are shown as mean ±SEM and a p<0.05 was considered significant.

Results

High traction forces correlate with less cell-dense and unstable monolayers
HUVECs were cultured on a traction force microscopy (TFM) set-up (Figure 1A) to study the distribution of contractile forces in resting cells. This analysis showed that the endothelium exerts heterogeneously distributed traction forces on the extracellular matrix (Figure 1B). The characteristic punctuated force
hot spots of which the magnitudes were several times bigger than the mean of the overall field, were found to be highly dynamic in time and location (Movie S1). Subsequent analysis revealed a negative correlation ($p=0.011$, $r^2 =-0.60$) between the monolayer cell density and the traction forces (Figure 2A), indicating that increased endothelial density reduces force generation within monolayers.

Next, we calculated force fluctuations, defined as the variation of normalized traction force over 15 consecutive time points/minutes. Highly dynamic monolayer areas were identified as regions with high forces fluctuations ($> 1.5 \times$ mean value per field of view (FOV)), as compared to areas with low force fluctuations ($< 0.5 \times$ mean value per FOV) (Figure 2B). We found that high traction forces correlate with high force fluctuations and thereby more unstable endothelial monolayers (44.7% co-localization), but not with low force fluctuations (0.4% co-localization). These data shows that HUVECs generate a heterogenic traction force landscape in which high contractile forces correlate with reduced cell density and more dynamic monolayers.

**Figure 1. Traction force microscopy reveals a heterogenic force landscape with characteristic punctuated hot spots.** (A) Contractile forces of human umbilical cord vein endothelial cells (HUVECs) were measured using traction force microscopy. In a glass bottom dish a polyacrylamide (PA) hydrogel was formed by polymerization which included a layer containing 2.0 µm (diameter) reference beads (indicated in blue), located between the glass and the substrate, and a densely packed layer of 0.1 µm top beads (in green). The top beads were directly cross-linked to the surface of the PA substrate and were used to visualize traction forces exerted by the cells on the matrix upon their displacement. The PA substrates were coated with collagen type I and had an elastic modulus of 1.2 kPa. At the end of the experiment, the cells were removed by trypsin and the unstrained gel substrate containing the beads was imaged. (B) Differential interference contrast (DIC) imaging revealed information about the confluency of the HUVEC monolayers. After drift correction, top bead displacements were calculated from the strained and unstrained fluorescent images and plotted in a displacement map. Analysis of the traction forces was subsequently
achieved by Fourier transformed traction cytometry and plotted in a traction force map. To gain insight into the localization of the traction forces relative to the cells, a merge of the DIC and traction force signals was made. Color bars represent the color-coded magnitudes of displacements in µm and traction forces in Pascal (Pa). All images were taken at 40x magnification and scale bars are 10µm.

Figure 2. High traction forces correlate with less dense and unstable monolayers. (A) Differential interference contrast (DIC) and VE-cadherin images reveal a certain variation in cell density (less-left vs more-right) of confluent endothelial monolayers on the traction force microscopy set-up. The middle graph represents the relation of monolayer traction forces with the cell density. Here, a reduction in root mean square (RMS) traction forces, which is a scalar measure of the cell's net contractile strength, was shown to correlate significantly (p=0.011, r² = -0.60) with the number of cells. The number of cells was assessed by counting the number of nuclei on the basis of the DIC images, ranging between 11 (±3.9x10⁴ cells/cm²) and 21 (7.5x10⁴ cells/cm²) per field of view (FOV). Each data point represents the mean per dish, which consist of 3 FOVs. For statistical analysis Spearman’s rank correlation coefficient was used. (B) The upper row of panels depict the DIC image of an endothelial monolayer, the corresponding traction map of forces higher than 1.5 times the mean (blue) and their merge. Subsequent green and red maps represent low (<0.5 x mean of the entire FOV) and high (>1.5 x mean) force fluctuations, respectively, which were defined as the variation of normalized traction forces over 15 baseline time points with time intervals of one minute and is a measure of monolayer stability. Co-localization of high traction forces and force fluctuations were plotted individually and as a merge of the indicated panels (orange). From the data points (52x52 per FOV, resolution of 2.5µm²) that showed high traction forces (>1.5 mean, blue), 0.4% correlated with low force fluctuations. In contrast, 44.7% of the high traction forces data points co-localized with high force fluctuations. Both experiments were conducted on 3 pools of 3 HUVEC donors. Scale bars indicate 10µm.
Contractile forces align with the F-actin cytoskeleton

F-actin is the main force-bearing cytoskeletal element in cells. To test whether F-actin organization is related to the orientation of contractile forces, F-actin stained HUVECs were analyzed by TFM. First, we established that the VE-cadherin antibody or the SiRactin compound did not affect the endothelial barrier (Figure SI). For the evaluation of orientation, force vectors of traction hotspots (> 2 x mean value per FOV) were superimposed on the traction force maps and the F-actin images. This showed that arrows within individual hotspots show primarily parallel orientation (Figure 3A(I)). A substantial part of these vectors aligned with the F-actin cytoskeleton (Figure 3A(II)), albeit that a subset of vectors oriented perpendicular to F-actin (Figure 3A(III)). Quantification of the offset (degrees) of the traction force arrows showed that 80% of these forces was oriented within a 45° angle of nearby F-actin cables (Figure 3B).

This high degree of correlation was also found for protrusions of the cortical F-actin ring, imaged in LifeAct-GFP-transfected endothelial cells. Filopodium formation was initially not associated with contractile forces (Figure 3C, t=0). However, during filopodium maturation and expansion, marked traction forces were detected, which reduced upon filopodium retraction (t=6 min). Similarly, contractile forces parallel the direction of a lamellipodia-like protrusion (Figure 3D). Moreover, force hotspots generating a constant traction force signal can be observed of which the forces were transmitted via thick, stable F-actin fibers. These data indicate that within an endothelial monolayer, traction forces correlate with either cortical F-actin bundles or with F-actin stress fibers.
Figure 3. Force hotspots show parallel vectors which align with the F-actin cytoskeleton. (A) DIC and fluorescent images of the F-actin cytoskeleton stained with SiRactin (Spirochrome) were obtained at 40x magnification. Traction force maps and F-actin merges were plotted such that only high traction forces (> 2 x mean) are shown as vectors (arrows). Force hotspots show parallel force vectors (I) the majority of which line up with the F-actin cytoskeleton (II). In addition, a subset of force vectors run perpendicular to F-actin (III). (B) Quantification of data presented in A was achieved by the introduction of a segmented grid to the total field of view (FOV). Per segment, a single comparison was made between the alignment of the traction force signal with the F-actin cytoskeleton. The offset (degrees) of the traction arrows was subsequently plotted as average incidence (percentages) of three independent experiments. (C-D) Representative images of force vector alignment with the F-actin cytoskeleton, visualized by the expression of LifeAct-GFP. (C) Newly formed filopodium (red arrows) which is protruding from the periphery of the cell generates traction forces during growth. Subsequently, retraction of the filopodium (time point 6 min) is associated with reduced traction forces. (D) The indicated lamellipodia-like protrusion (in blue) is transported alongside the cortical actin ring and generates traction forces in the direction of movement. In contrast, a stabile force hotspot (indicated in red) displays force vectors which align with thicker F-actin bundles within the cell body. Plotted force vectors are spaced 2.5µm apart. Scale bars indicate 10µm.

Contractile forces are high in the cell periphery
To investigate subcellular distribution of traction forces we stained cell-cell junctions with VE-cadherin anti-bodies and marked the nuclei based on DIC imaging (Figure 4). From the plotted traction force vectors over the individual VE-cadherin (a) and nuclear masks (b), it became clear that stronger forces were present near cell-cell junctions and lower forces were present underneath the nuclei. Cell-cell junctions presented significantly higher (+12.1%, p= 0.012) traction forces as compared to the nuclear areas (-12.1%, p= 0.012), whereas the cytosolic compartment was level with the mean traction forces of the FOV. Junctional regions which consisted of than two cells were additionally associated with higher traction forces (Figure SII: Tri-cellular corners vs junctional regions: +16.3%±3.5, p=0.044).

To test the force distribution in activated endothelium, we stimulated HUVECs with thrombin for 30 minutes and assessed the traction force distribution (Figure SIII). As a consequence of the stimulation, overall traction forces increased and inter-endothelial gaps were formed. Like in resting cells, sub-junctional forces were higher than sub-nuclear forces. In contrast, traction forces underneath the remaining cell body were lower than in the unstimulated condition (-12.9 vs +2.1% respectively), while forces in the thrombin-induced gap area were higher (+7.0%, ns) than the average forces of the FOV. Thus, individual cellular compartments can be discriminated on the basis of their specific traction force profiles.
Figure 4. Higher traction forces co-localize with cell-cell junctions whereas lower forces associate with the cell nucleus. Live HUVECs on the traction force microscopy set-up were stained for VE-cadherin using anti-human VE-cadherin Alex-647 anti-bodies, shown in green. Simultaneously, DIC images of the cells were captured and used to define the nuclei, encircled in blue. Traction force vectors plotted over the individual VE-cadherin (a) and nuclei masks (b) showed higher forces and therefore longer vectors at the cell-cell junctions and lower forces below cell nuclei. This data was quantified by dividing the traction forces of the total field of view (FOV) into three compartments; forces underneath the cell-cell junctions (green), the nuclei (blue) and the cytosol (white). Data points represent averaged traction forces per cell compartment (%) which showed to be significantly enhanced at cell-cell junctions and reduced at cell nuclei, although the cytosolic compartment was not altered compared to the mean of the total FOV. 3 pools of 3 HUVEC donors used in this experiment. Statistical significance was analyzed using an one-way ANOVA with Bonferroni post-hoc testing (** p<0.01). Scale bars indicate 10µm.
Thrombin induces high contractile forces and inter-endothelial gaps in unstable monolayer areas

Stimulation with thrombin caused an increase in contractile forces reaching a plateau after 10 minutes (Figure 5A) and remaining high, 30 minutes after the administration of thrombin (89.7±10.3 vs 36.2±12.4 Pascal in the control, p=0.02). Higher traction forces (> 2 x mean value per FOV) were non-randomly distributed over the cell-cell junctions (Figure 5B). Furthermore, we found that thrombin increased the size of force hotspots (100%±0.0 vs 446.5%±62.8, p=0.0015). This was associated with formation of inter-endothelial gaps, consequent to asynchronous and sometimes directionally opposing forces. Opening of intercellular gaps at t=10 minutes was followed by high contractile forces at the gap boundaries with low contractile force in the center of the gap.

Amplitude and stability of traction forces detected prior to stimulation were analyzed for their capacity to predict intercellular gaps upon stimulation. To do so, each gap origin was scored for high average traction forces (>1.5 x the mean) and high force fluctuations (>1.5 x the mean) and corrected for random appearance of gaps. Based on a significant (p=0.002) difference in predicting gap-appearance, we concluded that high force fluctuations, rather than high forces, predict future thrombin-induced gaps (Figure 6). The chance of gap formation in an unstable area without high contractile force is 4.9 times higher than random. In contrast, the chance of gap formation in a stable region with high traction forces was only 0.6 times higher than random. Thus, traction force instability correlates better with agonist-induced formation of intercellular gaps than the magnitude of these forces.
Figure 5. Thrombin induces endothelial contraction resulting in inter-endothelial gaps. (A) Endothelial traction forces increased significantly upon stimulation with the vaso-active agent thrombin (1U/ml). This is in contrast to the vehicle control where no alterations were observed during the acquisition period of 45 minutes. These endothelium-generated traction forces were expressed as root mean square (RMS) traction forces ± SEM and were measured in 3 independent experiments with 3 pools of 3 HUVEC donors. Statistical significance was analyzed using a non-parametric Mann Whitney test (* p<0.05). (B) High traction forces (> 2 x mean) in thrombin-treated cells, indicated with yellow, 2.5µm-spaced vectors, were asymmetrically distributed over the VE-cadherin stained cell-cell junctions. Moreover, these force hotspots tend to co-localize with tricellular contacts as depicted in the right panel (white lines mark cell-cell contacts). (C) Representative endothelial monolayer in which, over a time period of 25 minutes, an inter-endothelial gap is formed in response to stimulation with thrombin. Force hotspots markedly increased in size from 10 minutes onwards as revealed by merges of DIC and traction forces. VE-cadherin anti-bodies were used to detect inter-endothelial gaps (yellow asterisk). The transition from asymmetrical forces over the cell-cell junctions to perpendicular forces that open the intercellular gap occurs between 5-15 minutes. When the cell-cell contact is disrupted, an area of low traction forces is formed which is surrounded by high force peaks on the edges of the gap. Scale bars indicate 10µm.
Figure 6. High force fluctuations are a better predictor of gap formation than high traction forces. (A) All three depicted force-fluctuation maps combine information of traction forces and force fluctuations prior to thrombin stimulation, with images of the endothelial cells after the indicated time of treatment (in min) with thrombin. The predictive value of high traction forces (>1.5 x mean, blue), high force fluctuations (>1.5 x mean, red) and their co-localization (orange), for inter-endothelial gap formation was studied. Force fluctuations were defined as the variation of normalized traction forces over 15 minutes in untreated cells and represent monolayer stability. Inter-endothelial gaps were identified at their first appearance on the basis of the DIC time-lapse movies. Gap origin was determined by the center of the indicated gap of which the color corresponds to previously described force fluctuation and traction force values of its origin. In total, 73 gaps were analyzed in three independent experiments of HUVECs pools.

(B) Expresses the chances of a gap to be formed in a particular monolayer portion (high (Yes) vs low (No); traction forces or force fluctuations), relative to the expected change on the basis of its occurrence within the field of view. This shows, in combination with a significant difference in predicting gaps appearance (McNemar test; p=0.002), that high force fluctuations are a better marker for the formation of inter-endothelial gaps. Scale bars indicate 10µm.
Discussion
The main finding of this study is that fluctuating traction forces are a better predictor than force magnitude of inter-endothelial gaps induction by a pro-inflammatory mediator. Moreover, these dynamic forces were high below junctional areas, correlating with F-actin, and low below nuclei. These results highlight the importance of contractile forces in regulating the semi-permeable barrier properties of the endothelium.

Previously, our lab showed that confluent endothelial cells exert more tractions forces than single endothelial cells. In the current study, we show that under non-stimulated conditions, endothelial cell density within a monolayer correlated inversely to contractile force. It is important to note that this observation was made within the variation of our confluent monolayers (3.9x10⁴ cells/cm² and 7.5x10⁴ cells/cm²), which corresponds to the cell densities normally measured on regular culture plastics. This finding is in line with the previous observation that subconfluent monolayers express more force-transmitting integrin complexes compared to confluent monolayers which may serve to facilitate cell spreading to establish cell-cell contacts.

We found that high traction forces correlated with instable monolayer areas. Earlier studies showed that in non-endothelial cells, stronger contractile forces were exerted by short-lived unstable focal adhesions and that vinculin, a focal adhesion protein, stimulates the exertion of strong traction forces, whereas it hampers growth of the complex. However, the notion that approximately half of the total forces within the monolayer is distributed via VE-cadherin based intercellular junctions, suggests that neighboring cells play an important role in balancing tension. It is important to note that higher force fluctuations localize in close proximity of the cell-cell contacts. Thus, these findings support the idea that higher forces are exerted by immature, instable focal adhesions, but to draw such conclusion, live-cell staining of focal adhesion proteins will be required.

To address also cell-cell transmitted forces, we analyzed the orientation of the traction force to F-actin, being the most important force-bearing structure. We were able to document alignment of the traction force vector angles with the F-actin, which, like in in vivo and in vitro studies, was mainly organized in cortical bundles. The small subset of forces vectors that did not align parallel to the cytoskeleton is presumably due to the detection limitations of thin F-actin bundles within our applied live cell imaging method and other force-bearing cytoskeleton elements. In fast protruding filopodia and lamellipodia-like structures, traction forces correlated with the time-dependent growth, retraction and transport of these specific actin structures, like previous studies showed in single cells. Together, our data show that in a monolayer of cultured HUVEC the contractile forces align with the F-actin cytoskeleton, independent of its appearance in actin stress fibers, cortical bundles, filopodia or lamellipodia. Moreover, we found that junctional regions correlated with stronger contractile traction forces, whereas reduced forces were measured underneath the nuclei. This is in line with the previously reported distribution profiles of the traction force transmitting focal adhesions in confluent cells.

In contrast to non-stimulated conditions, thrombin acutely induced visible alterations of cell morphology, which matched the reported maximal intercellular Ca²⁺ (<30 sec) and MLC phosphorylation (60 sec). However, the build-up of marked traction forces required more time,
indicating the importance of additional downstream signaling of the heterotrimeric G-protein driven signaling in this process. The full traction force potential was reached after 10 minutes, which was in line with responses of single HUVECs. Earlier studies using an isometric force apparatus measured faster response times (<5 minutes). Despite these time differences, the observed contractile plateau phase of a more than two-fold increase of basal traction stresses was comparable and in line with more recent TFM studies. This stable period was sustained throughout our registration, and others have shown that this can persist for more than 1 hour. The endothelial cell activation evoked by thrombin has previously been linked to the tension-induced formation of inter-endothelial gaps. Here, we confirm this relationship and additionally present a detailed overview of the time-dependent formation of local monolayer gaps in overlap with the local traction forces and VE-cadherin expression. The loss of VE-cadherin based cell-cell contacts was preceded by the induction of highly heterogeneous and asynchronous monolayer forces, which seems to preferentially occur at tri-cellular corners. Within epithelial cell pairs, it was already suggested that tethering cell-cell forces are heterogeneously distributed over the junctions and favorably locate at peripheral vertices which express higher levels of cadherin, F-actin and myosin. Previously it was shown that junctional breakage itself, by the introduction of VE-cadherin blocking antibodies was accompanied by a decrease in overall monolayer contractions and thereby did not contribute to the thrombin induced increase in traction forces.

Recently, increasing studies reveal the importance of intracellular forces, which can be determined using intracellular stress microscopy. Assessing to what extent our current data on traction force distribution relate to intracellular stresses is beyond the scope of this study. However, the spatial correlations we describe between traction forces and cell junctions and F-actin fibers are not found in intracellular stress experiments. This can be either because the methods differ so much that within these experiments no location-dependent forces can be detected, or the differences are present but are directly balanced within the cytoskeleton and can no longer be observed at the level of the cell-cell junctions. However, due to the strong linear correlation between the cell-matrix and cell–cell forces in cell pairs, it seems less obvious that heterogeneous traction forces will result in homogenous intracellular stresses.

In conclusion, this study shows that the heterogeneous force landscape underneath the endothelium can be divided in nuclear areas were lower- and cell-cell junction areas where higher contractile forces are exerted. The formation of newly formed inter-endothelial gaps, following the administration of the vaso-active agent thrombin, was characterized by the generation of opposing traction forces at the gap margins. Finally, our data indicates that monolayer force stability is a key important feature controlling endothelial barrier dynamics and hyperpermeability.
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Disclosure
The authors declare no conflict of interest
References


Supplemental material
Detailed methods

Cell culture

Umbilical cords of healthy donors were provided by the Department of Obstetrics from the Amstelland Hospital (Amstelveen, The Netherlands) in accordance with the principles outlined in the Declaration of Helsinki. Isolation of human umbilical cord vein endothelial cells (HUVECs) was achieved as described previously. HUVECs were cultured on gelatin-coated culture plates in medium 199 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (#DE17-602E), 2 mM L-glutamine (#BE17-605E), (all Lonza, Verviers, Belgium), 5 U/ml heparin (#013192-03; Leo Pharmaceutical Products, Breda, The Netherlands), endothelial cell growth factor (crude extract from bovine brain), 10% heat-inactivated human serum (#34005100; Invitrogen, Brown Deer, WI) and 10% heat-inactivated newborn calf serum (#BE17-605E; Lonza). The cells were maintained at 37°C and 5% CO2 and medium was changed every other day. For all experiments pools of HUVECs of at least 3 donors in the second passage were used.

Preparation of polyacrylamide gel substrates

Polyacrylamide hydrogels with collagen ligation and embedded fiducial were produced as previously described with some minor adjustments. In brief, uncoated 20 mm glass bottom dishes (#P35G-0-20-C; MatTek Corporation, Ashland, MA) were treated with 0.1 M NaOH for 1 hour and subsequently soaked in distilled water. After air drying, a thin film of 97% 3-Aminopropyltriethoxysilane (APES, #281778; Sigma Aldrich, Steinheim, Germany) was added to the bottom glass and incubated for 3 minutes. Subsequently, the dishes were washed extensively in distilled water. Next, to the glass, 2 µm reference beads (1:1875 in water, #F8824; FluoSpheres®; Molecular Probes, Eugene, OR) were cross-linked with 0.5% glutaraldehyde (Fluka, St. Gallen, Switzerland) in PBS. After 30 minutes of cross-linking and three subsequent washing steps, 24 µl of an acrylamide (5.5%, #1610140), BIS (0.05%, #1610142), TEMED (0.05%, #1610801; All BioRad, Veenendaal, The Netherlands) and 0.5% of ammonia persulfate (APS, # A3678; Sigma Aldrich, Saint Louis, MO) mixture was added directly to the center of each dish. Before polymerization the gel solution was covered by a 18 mm coverslip (#CS18100; Menzel, Braunschweig, Germany) to create a flat and even surface. This procedure resulted in a compliant hydrogels of 1.2 kPa (Young’s modulus) as described previously with a final thickness of ~100 µm, as determined by the microscopic difference between top and reference beads.

After polymerization, the coverslip was removed and 200 µl of 1 mM sulfo succinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate (Sulfo-SANPAH, #22589; Thermo Scientific, Rockford, IL) in 0.1 M HEPES, pH 8.5, was added to the surface and activated by ultraviolet light for 3 minutes. Next, 200 µl of 0.2 µm sulfated top beads in water (1:200, #F8848; FluoSpheres®; Molecular Probes, Eugene, OR) was added and incubated for 20 minutes to achieve binding. Afterwards, the Sulfo-SANPAH treatment and top bead administration was repeated once more to achieve optimal bead density, followed by coating of the PA gels overnight at 4°C with 0.1 mg/ml purified type I bovine collagen (#5005-B, PureCol®, Advanced BioMatrix, Carlsbad, CA) in 0.1M HEPES. The subsequent day, the excessive coating was removed by washing twice with a 0.1 M HEPES solution, after which the gels were incubated for 30 minutes with M199. After aspiration of the M199, 50 µl of a concentrated cell suspension of HUVECs was directly added to the center of each PA gel. To this cell suspension, an additional 2 ml of
supplemented M199 was carefully added after 20 minutes of cell incubation. Afterwards, the HUVECs were grown for two consecutive days to confluence, in which the medium was refreshed once.

**Traction force microscopy**

The confluent HUVEC monolayers that were plated upon the polyacrylamide substrates were washed once and then pre-incubated for 1 hour in medium 199 supplemented with 1% human serum albumin (HSA; Sanquin Blood Supply, Amsterdam, The Netherlands). During this pre-incubation period, adherens junctions or the F-actin cytoskeleton were stained using the VE-cadherin staining antibodies (1:250, #561567; clone 55-7H1, BD Pharmingen, San Diego, CA) or 0.1 uM SiRactin (#SC001; Spirochrome, Stein am Rhein, Switzerland), respectively. The only exception made was for experiments depicted in Figure 3, were the F-actin cytoskeleton was visualized by lentiviral transfection with the inert LifeAct-mCherry 2 days prior to the experiments as previously described. The stained cells were visualized using a Zeiss Axiovert 200 MarinasTM wide-field inverted microscope that was equipped with a climate-controlled universal heating- and gas incubation system (Ibidi, Planegg, Germany; temperature: 37°C, CO2: 5%, humidity: 80%). Image acquisition was performed using a 40x Zeiss air objective (Carl Zeiss, Jena, Germany). Cell morphology was imaged in differential interference contrast (DIC), and the VE-cadherin or F-actin, in combination with top- and reference beads for the computation of traction forces were imaged using fluorescence. Slidebook software (Intelligent Imaging Innovation, Denver, CO) was used to control the minute interval time-lapse experiment. From each dish three regions of interest were selected, at least one field of view apart, from which alternately images of the cells, top beads and reference beads were taken. After a baseline period of 15 consecutive images, the HUVECs were stimulated with 1 U/ml of the vaso-active agent thrombin and followed for another 30 minutes. The last step consisted of the trypsinization of the endothelial monolayer from the substrate to acquire an unloaded fiducial bead pattern, after which a final image of all positions was captured.

**Computation of traction forces**

In order to determine monolayer traction forces, we used the well-established method of monolayer traction microscopy. Briefly, all images were cropped and corrected for small x-y drifts on the basis of the fixed reference bead pattern on the bottom of the glass dish. From the de-drifted images, gel displacements were calculated by comparing the pattern of substrate crosslinked tracer beads from each time point with the unloaded substrate situation after trypsinization of the cells. This was accomplished using particle image velocimetry (PIV) implemented at a spatial resolution of 2.5µm². From the displacement field and with the prior knowledge of the substrate material properties (substrate stiffness =1.2 kPa, Poisson’s ratio=0.48), the monolayer traction forces were calculated using constrained two-dimensional fast Fourier transformation method. From the monolayer traction fields, we calculated the root mean squared value of traction in Pascal, which is a scalar measure of the cell’s net contractile strength.

**Electric Cell-substrate Impedance Sensing (ECIS)**

Electrical impedance was used to detect functional effects of the used life-cell staining’s and was measured as previously described by Szulcek et al. with the use of an ECIS system (Applied Biophysics,
Troy, NY). In brief, HUVECs were seeded in 1:1 density on a 0.1 mg/ml collagen type I-coated ECIS arrays, each containing 8 wells with 10 gold electrodes per well. After culturing the HUVECs for 2 consecutive days, the cells were serum-starved for 90 minutes in 1% HSA in M199 and subsequently pre-incubated with the staining compounds in different concentrations as indicated. After 2.5 hours, the effect of the compounds on the thrombin-induced drop in barrier resistance was studied by adding thrombin directly to some wells in a final concentration of 1U/ml.
Supplemental References


Supplemental figures

Figure SI. Endothelial function was not affected by VE-cadherin and F-actin staining. Electrical impedance measurements of HUVECs were used to detect functional effects of the used live-cell stainings. The effect of the addition of staining solution was monitored under basal conditions in (A+C) and influence on the thrombin response was represented in (B+D). The VE-cadherin staining anti-bodies (55-7H1, BD Pharmingen) did not have any marked effects on basal endothelial resistance. Only after the administration of thrombin it seems to mildly reduce the thrombin-induced drop in the highest staining concentration (1:100). In contrary, SiR-actin (Spirochrome), used to stain F-actin, did show already under basal conditions a clear concentration-dependent effect on the ECIS readings. In concentrations of 1-0.1 uM SiR-actin affected the endothelial barrier negatively, however within the previously described experiments only a concentration of 0.05 µM was used. For experimental staining purposes of VE-cadherin, an anti-body dilution of 1:250 was used. Both experiments were conducted on 2 pools of 3 HUVEC donors.
Figure SII. Thrombin stimulation does not alter the relative force distribution over the monolayer. Traction force vectors plotted over the individual VE-cadherin (a) and nuclei masks (b) obtained using the traction force microscopy set-up in combination with the same staining techniques as described in Figure 4. After 30 minutes of thrombin stimulation the absolute traction forces were markedly increased, but the relative averaged traction force per cell compartment did not change compared to baseline (Figure 4). Higher traction forces and therefore longer vector arrows were co-localized with cell-cell junctions whereas lower and shorter vectors co-localized with cell nuclei. Although the trends were comparable to the baseline situation, this relation did not reach statistical significance due to higher variance. The newly introduced gap category of inter-endothelial gaps (white asterisks) was scored on the basis of the VE-cadherin cell borders in combination with the absence of a cell body in the DIC images and shown to have slightly higher average traction forces compared to the total field of view (FOV). Represented data points show the averaged value per dish with the mean, for three independent experiments with HUVEC pools. Statistical significance was analyzed using an one-way ANOVA with Bonferroni post-hoc testing (ns). Scale bars indicate 10µm.
Figure SIII. Model of the thrombin-induced contraction in relation to endothelial permeability. The time course of development of traction forces in the experiments in Figure 5A were correlated to changes in electrical impedance of HUVECs using ECIS (Applied Biophysics, Troy, NY). During the baseline period of 15 minutes, a stable endothelial cell monolayer was monitored in which normal values were considered to be around 40 Pa for root mean square (RMS) traction forces and 1200 Ohm for electrical resistance readings. This situation of low contractile force in combination with high barrier resistance was disrupted by the administration of 1 U/ml of thrombin. Immediately after the stimulation and even before traction forces and resistance was affected, endothelial cell deformation could be observed by DIC imaging. Within a minute, a strong induction of contractile traction forces is initiated which is accompanied by a drop in electrical monolayer resistance. Soon after this initial response, polymerization of F-actin can be observed which is followed by the formation of inter-endothelial gaps, of which the first were visualized around 7.5 minutes after thrombin stimulation. After 15 minutes, the traction force signal reaches its plateau phase whereas the resistance signal is still decreasing. In the last phase, gap closure can already be observed by the formation of lamellipodia, although traction forces are still high. In control experiments (i.e. experiments carried out without any cells) the traction force signal is around a 5-fold- and electrical impedance 4-fold lower.
Movie SI. Dynamic force landscape under basal and agonist-enhanced contractility. Time series of differential interference contrast (DIC) images of the cells (left), traction force maps (middle) and traction force merges (right) for a period of 45 minutes with a time interval of 1 minute between each frame. The first 15 consecutive images represent the basal situation where after the inflammatory agonist thrombin was introduced and followed for 30 minutes. Scale bars indicate 10µm.