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Research Article

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 acto-myosin-based contractile forces in the context of cell-cell and cell-matrix interactions. However, the spatio-temporal 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.

1. 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 Ca^{2+} levels, [2,7] and phosphorylation of myosin light chain and junctional proteins [4,6,8,9]. 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 places at cell-cell junctions through vascular endothelial cadherin (VE-cadherin), [13–15] which regulates vascular barrier function in vitro and in vivo [16–18] 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

Abbreviations: DIC, Differential Interference Contrast; F-actin, Filamentous actin; FOV, Field of View; HUVECs, Human Umbilical Vein Endothelial Cells; PA, Polyacrylamide; Pa, Pascal; RMS, Root Mean Square; TFM, Traction Force Microscopy; VE-cadherin, Vascular Endothelial cadherin

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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] spreading [26] and migration [27] and respond to mechanical cues such as fluid shear stress, [28,29] substrate stiffness [30] and stretch [31]. Moreover, traction forces are induced by age-associated changes [32] 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.

2. Material and methods

2.1. 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 [35,36]. Polyacrylamide hydrogels with collagen ligation and embedded fiducial markers were produced as previously described [30,37]. Subsequently, a concentrated cell suspension of HUVECs was directly added to the center of each gel and grown for two consecutive days to confluence.

2.2. 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, CO_2: 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, [24] with the prior knowledge of the substrate material properties (substrate stiffness = 1.2 kPa, Poisson's ratio = 0.48) [30]. 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.

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Soft-ware, San Diego, CA, USA). Statistical significance of the data represented in Fig. 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 Fig. 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.

3. Results

3.1. High traction forces correlate with less cell-dense and unstable monolayers

HUVECs were cultured on a traction force microscopy (TFM) set-up (Fig. 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 (Fig. 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 SI). Subsequent analysis revealed a negative correlation ($p = 0.011$, $r^2 = -0.60$) between the monolayer cell density and the traction forces (Fig. 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) (Fig. 2B). We found that high traction forces correlate with high force fluctuations and thereby more unstable endothelial monolayers (44.7% co-localization) ($> 1.5 \times$ mean value per FOV), 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.

3.2. Contractile forces align with the F-actin cytoskeleton

F-actin is the main force-bearing cytoskeletal element in cells [38]. 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 (Fig. SI). For the evaluation of orientation, force vectors of traction hotspots ($> 2 \times$ 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 (Fig. 3A(I)). A substantial part of these vectors aligned with the F-actin cytoskeleton (Fig. 3A(II)), albeit that a subset of vectors oriented perpendicular to F-actin (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3D). Moreover, force hotspots generating a