Regulation of endothelial barrier function during flow-induced conversion to an arterial phenotype

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Abstract

Objective: Flow-induced conversion of endothelial cells into an elongated arterial phenotype requires a coordinated regulation of cell junctions. Here we investigated the effect of acute and chronic flow on junction regulation.

Methods and results: Using an extended experimental setup that allows analyses of endothelial barrier function under flow conditions, we found a flow-induced upregulation of the transendothelial electrical resistance within minutes. This was accompanied by an increase in actin filaments along the junctions and vascular endothelial (VE)-cadherin clustering, which was identified at nanoscale resolution by stimulated emission depletion microscopy. In addition, a transient tyrosine phosphorylation of VE-cadherin and catenins occurred within minutes following the onset of flow. VE-cadherin and actin distribution were maintained under chronic flow over 24 h and associated with the upregulation of VE-cadherin and α-catenin expression, thus compensating for the cell elongation-mediated increase in cell border length. Importantly, all observed effects were rac1 dependent as verified by the inhibitory effect of dominant negative N17rac1.

Conclusion: These results show that flow-induced conversion of endothelial cells into an arterial phenotype occurs while intercellular junctions remain intact. The data place rac1 in a central multimodal regulatory position that might be important in the development of vascular diseases, such as arteriosclerosis.

Keywords: VE-cadherin; Actin; Rho-GTPases; Cell junction; Permeability

1. Introduction

Fluid flow plays a pivotal role in vascular endothelial cell structure and function. Endothelial cells of the veins are polygonal in shape, while endothelial cells of the arteries are spindle shaped and align in the direction of flow [33]. The conversion of endothelial cells into an arterial phenotype in response to flow requires a complex coordination of mechanosensing [10,30,50], intracellular signalling [19], early and late cytoskeleton rearrangements [5,21,35,43,48] and
differential gene expression [11,23,51]. The flow-induced conversion of the endothelial phenotype follows a characteristic time course in association with cell alignment and cell migration [15]; however, spatial and temporal remodelling of inter-endothelial junctions and the underlying mechanisms of this process remain to be investigated.

The vascular endothelial (VE) cadherin/catenin complex plays a critical role in the regulation of endothelial cell adhesion and barrier function [3]. The extracellular domain of VE-cadherin directly connects adjacent cells to each other while the intracellular domain binds either $\beta$- or $\gamma$-catenin and p120, a protein that appears to stabilize cadherins and regulate cell motility [3,55]. It was proposed that binding of cadherin/$\beta$-catenin to cortical actin filaments [41] via $\alpha$-catenin enhances cadherin-mediated cell adhesion. However, recent data show that rather than directly providing a link to actin filaments, $\alpha$-catenin appears to function as a key molecule in regulation of actin dynamics [17].

The VE-cadherin/catenin complex and the junction-associated cortical actin filaments are targeted by many signalling pathways that are critical for junction regulation [3,55,59]. Tyrosine phosphorylation/dephosphorylation has been implicated in the modulation of VE-cadherin localization, cell adhesion and barrier function following stimulation of endothelial cells with growth factors, phosphatase inhibitors or inflammatory mediators [1,2,18,47]. Rho-GTPases, particularly rhoA, rac1 and cdc42, have also been shown to be important players in the regulation of actin dynamics [25,59] and the modulation of cadherin-mediated cell adhesion [32]. Activation of rhoA by thrombin leads to stress fibre formation [59] resulting in reduced endothelial barrier function. In contrast, rac1 activation appears to increase the localization of cadherins at cell junctions, to promote junctional integrity [59]. Recently, a cross talk between rac1 and tyrosine phosphorylation of the VE-cadherin/catenin complex was reported [47,53,54] indicating a functional connection. Although shear stress mediates rho-GTPase activity [51], which contributes to cell migration, cell alignment, actin reorganization and activation of transcription factors [51,60], a functional role for the regulation of paracellular barrier function under flow conditions has not been shown so far.

Here we performed a comprehensive study to analyse the regulation of endothelial barrier function during flow-induced conversion of endothelial cells into an arterial phenotype.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal antibodies directed to VE-cadherin, $\alpha$-,$\beta$- and $\gamma$-catenin, p120 and anti-phosphotyrosine (horse-radish peroxidase labelled) were from Transduction Laboratories (Dianova, Hamburg, Germany). PECAM-1 was from R&D Systems GmbH (Wiesbaden, Germany). Appropriate secondary antibodies were either from Molecular Probes (Göttingen, Germany) or Dianova (Hamburg, Germany). Phosphotyrosine antibody pt66, tetramethylrhodamine isothiocyanate (TRITC) labelled phalloidin and all other reagents were from Sigma (Deisenhofen, Germany).

2.2. Cell culture and immunofluorescence microscopy

Endothelial cells derived from arteries and veins of human umbilical cords were cultured as described elsewhere [42]. Cell purity was nearly 100% as tested by VE-cadherin labelling. For comparison of protein expression in arterial versus venous endothelial cells primary cultures (3 days culture) were used while cells from the first passage were used for the flow experiments. Cells were seeded in endothelial cell growth medium (Promocell, Heidelberg, Germany) on glass slides especially manufactured for impedance spectroscopy under flow [46]. The investigation conforms to the principles outlined in the Declaration of Helsinki for use of human tissue. Antibody labelling was performed as described elsewhere [43]. Immunofluorescence using phosphotyrosine antibody was performed in the presence of ortho-vanadate (1 mmol/l).

2.3. Shear stress experiments and impedance spectroscopy

Electrical impedance spectroscopy was used to determine the transendothelial electrical resistance (TER) under defined flow conditions [46] using a cone and plate rheological system described elsewhere [6,15,42,46].

2.4. STED microscopy

The STED microscope has been described previously [16]. In brief, fluorescence excitation was performed with a pulsed laser diode emitting 100 ps pulses at 470 nm (Picoquant, Berlin, Germany), while STED utilizes 280 ps pulses with a repetition rate of 250 kHz at 603 nm, generated by a opti-parametric amplifier (OPA) being pumped by a regenerative amplified Ti:Sapphire laser (Coherent, Santa Clara, CA, USA). The excitation and the STED beams were coupled into an oil immersion lens (HCX PL APO, 100x, Leica Microsystems, Mannheim, Germany) with a 1.4 numerical aperture, by means of dichroic mirrors. The average power of the excitation beam and the STED beam in the sample was 0.1 $\mu$W and 0.25 mW respectively. The fluorescence was collected by the same lens and directed into a counting avalanche photodiode (Perkin Elmer Optoelectronics, Canada).

Deconvolution in terms of a single-step LD, i.e., Wiener filter, was carried out where indicated (LD) with a theoretical PSF assuming a FWHM of 36 nm. Noise-induced negative values <10% of the image maximum were clipped.

2.5. Quantitative image analysis of cell border length and overlapping cell junctions

The ImageJ-Software (NIH) was used for quantitative image analysis. Cell borders stained for junctional proteins
were converted into a black and white image using an appropriate threshold value. The image was skeletonized and remaining, not junction associated, dots were cleared manually. The entire cell border length the cell number, the cell area and the cell perimeter were determined from those images. Areas of overlapping endothelial cell junction [24] were manually selected as illustrated in Fig. 2C (white lines around the overlapping cell area). The red lines in Fig. 2C mark linear junctions. The overlapping cell area was normalized to the total pixel number of the image and expressed as %.

2.6. Analyses of tyrosine phosphorylation by immunoprecipitation and Western blotting

Tyrosine phosphorylation of the respective proteins was analysed according to a protocol described elsewhere [47]. Briefly, the experiments were performed in Medium 199 (M199) supplemented with 10% FCS [42]. Sodium orthovanadate (1 mmol/l) was supplemented for 45 min, which had no effect on tyrosine phosphorylation by itself. Prior to immunoprecipitation total protein of Triton X-100 soluble and insoluble fractions were determined by Amidoschwarz method [14] and samples of different experiments were standardized such that they contained the equivalent total protein amount in the same volume. Immunoprecipitations and Western blot analyses were performed as described elsewhere [24,43,47].

2.7. rac1 pulldown assay

Rac1-activity was determined as recently described [47,60]. Briefly, a recombinant GST-p21 binding domain (PBD) of Pak-1 bound to glutathione beads (Amersham Biosciences) was used to pull down active rac1 proteins. The pulled down rac1 was resolved by SDS-PAGE and detected by Western blotting.

2.8. Adenovirus generation and gene transduction

Amplification and purification of replication deficient adenoviruses containing the sequence for the green fluorescent protein (GFP), dominant negative rac1 (N17rac1) or dominant negative rhoA (N19rhoA) were performed as described elsewhere [47,60]. HUVECs were infected with the respective adenovirus for 4 h, washed and cultured for a further 16 h. About 85±10% of the cells expressed GFP after infection with AdGFP and the TER remained constant under resting culture conditions.

2.9. Statistical analysis

Comparison between groups was performed by unpaired t-test. Results were considered statistically significant if \( p < 0.05 \).

3. Results

Laminar flow was applied to human umbilical cord vein endothelial cells (HUVECs) using an extended cone-and-plate rheological system that is equipped with an impedance spectroscopy setup [6,15,42,46]. Following equilibration under low flow (0.5 dyn/cm²), shear stress of 6, 12 or 30 dyn/
cm\(^2\) caused a flow-dependent, transient increase in TER where the slope and the magnitude of the TER increased in a shear stress dependant manner (Fig. 1A). Also, the TER decrease was flow dependent. It occurs earlier and was accelerated at higher flow levels (not shown). These data are in agreement with a recent report using porcine endothelial cells [46]. Over the next 24 h, cells continuously elongated and aligned in the direction of flow which was accompanied by a slow and moderate TER decrease, reaching a steady state level of 75±11% as shown for 12 dyn/cm\(^2\) (Fig. 1B). Both the flow-induced endothelial cell elongation and the TER decrease reversed within 24 h when the flow was scaled back to 0.5 dyn/cm\(^2\) (Fig. 1C).

Under control conditions immunofluorescence staining of VE-cadherin displays overlapping endothelial junctions [24] with poorly developed junction-associated cortical actin filaments (Fig. 2A). Flow (12 dyn/cm\(^2\)) reduced overlapping cell junctions quickly (within 20 min) and VE-cadherin was linearly distributed along the junctions in co-localization with junction-associated actin filaments (JAAF) (Fig. 2A, arrowheads). Even during flow-induced cell elongation VE-cadherin, β- and γ-catenin remained linear distributed along the junctions and JAAF persists as well (Fig. 2B, Supplementary data 1). During reversion (24 h 12 dyn/cm\(^2\) followed by another 24 h of 0.5 dyn/cm\(^2\)) the VE-cadherin linearization and the JAAF persist as well, and overlapping cell junctions do not reappear (Fig. 1C). Quantification of overlapping cell junctions by image analysis revealed a decrease from 12±6% under low flow to 6±1% under high flow (Fig. 2C). The data show a flow-induced early and quick tightening of endothelial cell junctions associated with VE-cadherin linearization and an increase in JAAF.

3.1. STED microscopy uncovered lateral clustering of VE-cadherin in response to flow

Classical immunofluorescence microscopy is limited to a resolution of about 200 nm. However, STED microscopy, a novel subdiffraction resolution microscopy technique [27] provides nanoscale optical resolution [16,26,58]. The STED setup simultaneously acquires images both in the confocal and the STED mode, with a focal spot size of 190 nm and ~30 nm respectively (Fig. 3A–F). This gives STED the ability to separate objects that are 30 to 40 nm apart in the focal plane [16]. STED imaging of endothelial VE-cadherin under low flow (0.5 dyn/cm\(^2\), 24 h) revealed round shaped clusters (50 clusters were analysed) of 63±11 nm in diameter that randomly distributed along the junctions (Fig. 3G). Consistent with the observed TER increase and the reorganization of junctional proteins and f-actin, STED

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Fig. 2. Reorganization of VE-cadherin and actin under shear stress. (A) Cells were exposed to low (0.5 dyn/cm\(^2\)) or high flow (12 dyn/cm\(^2\)) for 20 min and stained for VE-cadherin and actin filaments by phalloidin rhodamine. Under low flow, VE-cadherin appeared as an irregular network displaying large overlapping and linear junctions (A, arrows) partially in colocalization with actin filaments (A, arrows). Application of flow (12 dyn/cm\(^2\)) increased the actin filaments at the cell junctions within 20 min (A, arrowhead), and was accompanied by a reorganization of VE-cadherin into an overall linear pattern (A, arrowhead). (B) Linearized VE-cadherin and JAAF distribution remained stable even after 24 h of flow at 12 dyn/cm\(^2\). (C) Overlapping cell junction areas were quantified by image analysis. The images illustrate linear (red lines) and overlapping junction areas (encircled by white lines) under low and high flow conditions (here shown for γ-catenin staining).
microscopy revealed a largely linear distribution of VE-cadherin after 15 min of shear stress exposure due to extended lateral clustering (Fig. 3B). The cluster size remains basically unchanged with a diameter of 60 ±11 nm at this time point. In contrast, after 24 h of high flow STED microscopy uncovered a further linearization of the cell junctions (Fig. 3C), featuring single chains of VE-cadherin clusters at the cell-to-cell junction. Consistent with this observation, the cluster sizes are significantly increased to 80±15 nm in diameter, indicating an aggregation of protein clusters at the elongated cell junction after 24 h of high flow exposure. In fact, further evidence of this long-term reorganization effect is given by the number of clusters per chain length, which are considerably reduced from 12 clusters per μm under low shear stress conditions to 6 clusters per μm for cells exposed to high shear stress for 24 h (Fig. 3H). Thus shear stress induced reorganization of VE-cadherin consists of a short-term linear reorganization of the VE-cadherin clusters, while long-term exposure also induces cluster aggregation at the cell contacts.

3.2. Flow-induced cell elongation is compensated for by increased expression of junction proteins

VE-cadherin clustering and an increase in JAAF in response to flow indicates stabilized cell junctions, regardless
of the moderate decrease in TER within 24 h. Thus, we explored if changes in geometrical parameters of the intercellular space can account for the moderate flow-induced decrease in TER. The three dimensional intercellular space is characterized by the parameters width, height and cell border length. Changes to one or more of these parameters will change the TER. The determination of the total cell border length of a given cell culture area before and after flow application of 12 dyn/cm² for 24 h revealed a flow-dependent increase in cell border length to 154 ±9% (Fig. 4A) that was due to the extension of cell perimeters by about 33% and a small increase in the cell number by about 15% (Fig. 4A). Thus, the flow-induced increase in cell border length increases the electrical current through the cell. Figure 4A shows the quantification of cell border length for a given culture area under different flow conditions. The length of the cell border increased by 53% for cells exposed to enhanced shear stress (12 dyn/cm²) for 24 h, while the TER was reduced by approximately 23%. Normalization of the TER to the length of the cell border showed an increase of about 20% after 24 h of shear stress. Increased cell border length under high flow was due to an increased cell number (15%) while at the same time the perimeter per cell was increased by about 33%. These effects were largely reversible, when shear stress was scaled back to 0.5 dyn/cm² for 24 h. TER(0) was 13 ±4 Ω cm² (*P<0.05; **P<0.01). Figure 4B shows Western blot analyses of VE-cadherin and α-catenin, PECAM-1 and β-catenin, or p120 and γ-catenin of cells exposed to low (0.5 dyn/cm²) and high (12 dyn/cm²) flow. All blots were additionally probed for α-tubulin to verify that the same amount of total protein was loaded. Enhanced expression of VE-cadherin, α-catenin and PECAM-1 for shear stress exposed cells was found (n ≥ 3). (C) Comparative Western blot analysis of VE-cadherin, α-catenin and PECAM-1 of endothelial cells derived from human umbilical cord arteries (Ar) and veins (Ve) shows increased expression of the respective proteins in arterial endothelial cells (n=4–6).
intercellular junctions and in turn reduces the TER. The flow-mediated cell elongation and TER decrease was reversible when the flow was scaled back to 0.5 dyn/cm² for another 24 h (Fig. 4A).

The moderate TER decrease appears to be less than the expected from the extension of the cell border length; therefore, we looked to see if the resistance of a defined length-segment of the intercellular space had been altered following flow application by normalization. According to a simple model [9] the TER of a given cell culture area (cm²) can be described as:

\[
\text{TER} = \frac{(\rho \cdot h)}{(w \cdot L)}
\]

(1)

where \( \rho \) is the resistivity of the bulk solution (medium, \( \Omega \) cm), \( h \) is the height (cm) and \( w \) is the width (cm) of the intercellular space. \( L \) (cm cm⁻²) is the total cell border length (cm) per cell culture area (cm²). To compare the relative resistance of the intercellular space before and after flow exposure, we normalized the TER (according to Eq. (2)). The resulting parameter describes the relative resistance of the intercellular space \( (R_i) \) which is independent of the cell border length.

\[
R_i := \frac{(\text{TER} \cdot L)}{(\rho \cdot h) / w}
\]

(2)

(derivation see Supplementary data 2)

Indeed, \( R_i \) increased by 20% in flow-adapted cells compared to control cells. Although this calculation does not allow conclusions about the individual changes of \( \rho \), \( h \) or \( w \) it further indicates that the moderate TER decrease is due to increased cell border length rather than an opening of the intercellular junctions.

3.3. Endothelial cells exposed to flow or isolated from arterial vessels display increased amounts junction molecules

To determine the mechanisms on how the intercellular barrier function is maintained during flow-induced cell elongation we explored the expression of the VE-cadherin...
and catenins. Indeed, there was an upregulation of VE-cadherin (55±24%), α-catenin (55±39%), PECAM-1 (112±53%) and β-catenin (21±27%) in flow-exposed endothelial cells within 24 h (Fig. 4B) while γ-catenin and p120ctn remained unchanged (Fig. 4B). While the cell elongation and the TER changes were reversible (Fig. 4A) the expression of VE-cadherin and α-catenin remained elevated (not shown).

To further assess the relevance of shear stress in the conversion to arterial phenotype we investigated the expression of junction proteins of primary cultures of arterial and venous endothelial cells isolated from umbilical cord vessels. Arterial endothelial cells exhibited a significantly higher expression of VE-cadherin (45±26%), α-catenin (19±19%) and PECAM-1 (47±27%) compared to venous endothelial cells, irrespective of a few days culture, as investigated by Western blotting (Fig. 4C). No significant differences were found for β-catenin, γ-catenin and p120ctn. Taken together, the data show that shear stress induced reorganization of endothelial cell junctions and an upregulation of junction proteins seems to compensate for the cell elongation but the overall resistance of a collective of endothelial cell area decreases slightly.

3.4. Rac1 activity is required for shear stress induced adaptation of endothelial barrier function

Rac1 has been shown to be critical in regulation of actin dynamics and cadherin organization [32,59]. Thus, we tested to see if rac1 was activated during flow-induced endothelial barrier function alterations. We found that 10 min after the onset of flow (12 dyn/cm²) rac1 was activated that persisted for 24 h (Supplementary data 3). The expression of a dominant negative rac1 (N17rac1), did not reduce the TER under resting conditions, but blocked the early and transient flow-induced increase in TER (Fig. 5A,B), the reorganization of VE-cadherin, the increase in JAAF, (Fig. 5C), cell elongation (Fig. 6).}

Fig. 6. Early effects of flow on tyrosine phosphorylation of junction proteins. Endothelial cells were exposed to different flow levels followed by immunoprecipitation of the VE-cadherin/catenin complex and western blot analysis using anti-phosphotyrosine antibody. (A) Low flow caused no tyrosine phosphorylation, while high flow (here shown for 30 dyn/cm²) resulted in an early (1 min) transient tyrosine phosphorylation of the VE-cadherin/catenin complex followed by dephosphorylation. (B) Analysed the time courses of transient tyrosine phosphorylation due to incremental flow application for 10 min shows an already decreasing tyrosine phosphorylation at higher flow rates while at 6 dyn/cm², the levels were still high. (C) Expression of dominant negative rac1 (N17rac1) blocked the flow-induced tyrosine phosphorylation of the VE-cadherin/catenin complex. (D) Immunofluorescence analysis of cells expressing (N17rac1) showed no enhanced tyrosine phosphorylation along the cell borders under flow compared to controls.
alignment (Fig. 5D) and the increase in VE-cadherin and α-catenin expression (Fig. 5E). In contrast, expression of either dominant negative rhoA (N19rhoA) or GFP had no influence on the shear stress-induced early and transient TER increase (Fig. 5B). These data indicate that rac1 activation plays a critical role in the physiological and dynamical regulation of endothelial junctions under flow.

3.5. Shear stress-induced transient tyrosine phosphorylation of VE-cadherin and catenins requires rac1-activation

Shear stress induces an early tyrosine phosphorylation of different proteins including MAPK [62], cortactin [5], proline-rich tyrosine kinase [49] and cytosolic β-catenin [52]. Here we investigated the effect of flow on tyrosine phosphorylation of the VE-cadherin/catenin complex. While low flow of 0.5 dyn/cm² had no effect (Fig. 6A), higher flow levels caused a flow-dependent early and transient increase in tyrosine phosphorylation of VE-cadherin, β-catenin and γ-catenin (Fig. 6A). We next tested if increased flow levels caused acceleration of the tyrosine phosphorylation-transient (the increase and decrease of tyrosine phosphorylation as a function of time). Thus, we exposed endothelial cells to incremental flow rates (6, 12 and 30 dyn/cm²) for 10 min. As demonstrated in Fig. 6B after 10 min the tyrosine phosphorylation of the VE-cadherin/catenin complex was highest at 6 dyn/cm² but had already decreased at flow levels of 12 or 30 dyn/cm² (Fig. 6A and B). This corresponds to the accelerated transient tyrosine phosphorylation at incremental flow rates. Consistently, immunofluorescence localization of tyrosine phosphorylated proteins in endothelial cells revealed a transient labelling at cell junctions and cytoplasmic proteins within minutes of the onset of flow with recovery to baseline levels within 30 min (Supplementary data 4).

To test the effect of rac1 on tyrosine phosphorylation we used high arterial flow levels of 30 dyn/cm² that causes the strongest reaction. The flow-induced effects of both the tyrosine phosphorylation of VE-cadherin and the overall labelling of tyrosine phosphorylated proteins at the junctions was completely blocked in cells expressing N17rac1 while tyrosine phosphorylation of β- and γ-catenin was only moderately reduced (Fig. 6C, D).

Taken together, the data place rac1 in a central role regarding the adaptation of endothelial barrier function to flow conditions by targeting multiple signalling mechanisms that result in VE-cadherin clustering, junction recruitment of actin, expression of junction molecules and tyrosine phosphorylation of VE-cadherin and catenins.

4. Discussion

In the present work we investigated the paraendothelial barrier function during flow-induced conversion into an arterial phenotype. The most significant findings of this study are that under flow conditions endothelial cell layers exhibit protective mechanisms that largely maintain intercellular integrity even during flow-induced cell elongation. In addition, we provide evidence that VE-cadherin-clustering and an increase in JAAF tightens cell junctions. This holds true for early and long-term flow exposure that cause dynamic reorganization of cell junctions within hours. Furthermore, rac1 activation was identified as a critical multimodal signal that is required for flow-induced cell alignment, increases the expression of VE-cadherin, α-catenin and PECAM-1 as well as the tyrosine phosphorylation of the VE-cadherin/catenin complex and is responsible for the maintenance of the endothelial barrier function under flow.

4.1. Dynamics of intercellular barrier function under flow

Onset of flow to arterial values (6 to 30 dyn/cm²) caused a dose dependent upregulation of the TER, which we interpret as a protective mechanism of the cell monolayer preventing cell dissociation and barrier function decrease due to flow. Analysis of the impedance spectra by an alternative model [34] confirmed this interpretation (Supplementary data 5). A TER increase in response to flow is also described for other endothelial cell types [13,46] and in response to stimulation by VEGF, cAMP or pervanadate [47,57]. Mechanistically, we hypothesize that the early TER increase reflects junction activation that allows flow-induced conversion of the cells into an elongated and flow-aligned phenotype. Cell alignment and cell elongation in response to flow occur within hours, reducing the flow-resistance and in turn the mechanical load of the cells [10]. However, the consequence of cell elongation is a TER decrease that seems to be compensated for by an increase in the expression of VE-cadherin, α-catenin and PECAM-1. Without this balancing of adhesion molecule expression in relation to cell border length, the intercellular adhesion and in turn the intercellular barrier function would be significantly disturbed. The flow-induced expression of VE-cadherin and catenins is in line with other reports [39], but appears to be dependent on the specific cell type and the culture conditions [36,52]. In our experimental system, an increased expression of VE-cadherin, α-catenin and PECAM-1 was observed in flow-exposed vein endothelial cells that is consistent with the increased expression of these molecules in endothelial cells which were isolated from human umbilical arteries that are typically exposed to high flow. The TER did not fully recover under chronic flow within 24 h; however, a full compensation of the intercellular tightness in response to cell elongation might take much longer as described for the JAAF in endothelial cells of the newborn rat aortas [31]. Moreover, persistence of flow was required to maintain cell elongation as the interruption of flow causes reorganization of the cells into a more polygonal phenotype accompanied by a TER increase. This observation further indicates that cell elongation, rather than down regulation of endothelial barrier function, is the cause of the moderate TER decrease under flow.
There is increasing evidence that the development of adherens junctions and tight junctions is linked [29,37]. Although HUVECs express tight junction proteins, they usually do not develop tight junction strands [20], but it was reported that flow induces the development of rudimentary tight junction-like structures in porcine aortic endothelial cells [63]. Interestingly, the tight junction protein occludin becomes phosphorylated under flow and it was proposed that this increased the hydraulic conductivity [12,40]. It has to be further explored how tight and adherens junction proteins coordinate under flow conditions to sufficiently maintain the endothelial barrier function.

4.2. Mechanisms of endothelial barrier function adaptation to fluid flow

Based on LSM and STED microscopy we provide evidence that VE-cadherin clustering, which was associated with linearization of VE-cadherin and increased localization of JAAF, is critical for adaptation and mechanical resistance of endothelial cells to flow. STED microscopy allowed the analysis of VE-cadherin organization at the 30 nm level. VE-cadherin is arranged as small clusters that are randomly distributed along the junctions and display a punctuated network of clusters at overlapping cell junctions. Flow, however, caused an early linearization of VE-cadherin by maintained cluster size while VE-cadherin clustering was seen after 24 h. This data show that endothelial cell junctions respond with an early, “emergency” measure followed by stable molecular rearrangement of VE-cadherin (clustering) under chronic flow conditions.

All the investigated parameters (TER changes, cell alignment, and tyrosine phosphorylation of VE-cadherin/catenins) were strictly rac1 dependent, indicating an essential and multimodal role of rac1 in this process. In particular the TER decrease in N17rac1-expressing cells indicates the critical importance of rac1 for cell junction activation. Beside the activation of rac1, flow induces a number of cellular reactions that might be important for dynamic rearrangement, e.g. an increase in intracellular [Ca²⁺] [45], phosphorylation of MLC [56], or production of NO [7] or ROS [28]. Activation of rac1 seems to be critical to allow these dynamics by maintained intercellular integrity. The time course of tyrosine phosphorylation of VE-cadherin and catenins appears to correlate with the slope of the flow-induced initial TER increase, we propose that tyrosine phosphorylation is a critical signal to allow cell junction reorganization by maintaining barrier function. This effect was completely blocked by N17rac1. The data are consistent with reports showing that an increase in tyrosine phosphorylation of junction proteins causes VE-cadherin reorganization, actin recruitment [2,47] and a transient increase in TER [47]. The presented data expand the role of rac1 in the adaptation process of endothelial cell layers to flow. It was shown that rac1 activation depends on integrin dynamics [51], which is critical for endothelial cell alignment of individual cells [60] and of confluent endothelial cell layers (present study) in response to flow. These mechanisms might be relevant in pathological processes, such as inflammation, leukocyte recruitment [61] and LDL-uptake [38]. These factors combined with insufficient endothelial barrier function contribute to the development of arteriosclerosis [8,22]. The development of atherosclerotic plaques occurs in regions of low and disturbed flow, while laminar shear stress is considered to be endothelial protective [4]. It is therefore tempting to assume that the herein described rac1-dependent stabilization of endothelial cell contacts in response to flow might contribute to atheroprotective effects in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.04.017.

References


