F-Actin–Anchored Focal Adhesions Distinguish Endothelial Phenotypes of Human Arteries and Veins

Daphne van Geemen, Michel W.J. Smeets, Anne-Marieke D. van Stalborth, Leonie A.E. Woerdeman, Mat J.A.P. Daemen, Peter L. Hordijk, Stephan Huveneers

Objective—Vascular endothelial–cadherin- and integrin-based cell adhesions are crucial for endothelial barrier function. Formation and disassembly of these adhesions controls endothelial remodeling during vascular repair, angiogenesis, and inflammation. In vitro studies indicate that vascular cytokines control adhesion through regulation of the actin cytoskeleton, but it remains unknown whether such regulation occurs in human vessels. We aimed to investigate regulation of the actin cytoskeleton and cell adhesions within the endothelium of human arteries and veins.

Approach and Results—We used an ex vivo protocol for immunofluorescence in human vessels, allowing detailed en face microscopy of endothelial monolayers. We compared arteries and veins of the umbilical cord and mesenteric, epigastric, and breast tissues and find that the presence of central F-actin fibers distinguishes the endothelial phenotype of adult arteries from veins. F-actin in endothelium of adult veins as well as in umbilical vasculature predominantly localizes cortically at the cell boundaries. By contrast, prominent endothelial F-actin fibers in adult arteries anchor mostly to focal adhesions containing integrin-binding proteins paxillin and focal adhesion kinase and follow the orientation of the extracellular matrix protein fibronectin. Other arterial F-actin fibers end in vascular endothelial–cadherin-based endothelial focal adherens junctions. In vitro adhesion experiments on compliant substrates demonstrate that formation of focal adhesions is strongly induced by extracellular matrix rigidity, irrespective of arterial or venous origin of endothelial cells.

Conclusions—Our data show that F-actin–anchored focal adhesions distinguish endothelial phenotypes of human arteries from veins. We conclude that the biomechanical properties of the vascular extracellular matrix determine this endothelial characteristic. (Arterioscler Thromb Vasc Biol. 2014;34:2059-2067.)

Key Words: arteries ■ focal adhesions

The luminal side of blood and lymphatic vessels is covered by an endothelial cellular monolayer that forms a selective barrier for solutes, immune cells, and other circulating cells. Increased vascular permeability during chronic inflammation often underlies the development of vascular disease.1–3 Moreover, during aging, when prolonged structural changes of the extracellular matrix (ECM) within the blood vessel wall cause stiffening of the arteries, enhanced endothelial monolayer permeability strongly contributes to the development of hypertension, atherosclerosis, and other cardiovascular diseases.4–6 Unfortunately, not many effective therapies are currently available to treat leaky vessels,7 and a better understanding of the endothelial phenotypes in human vasculature could provide new insights for treatment possibilities.

Endothelial cells make adhesive contacts to the ECM of the vascular wall as well as homotypic adhesion between neighboring cells. Both adhesion structures are essential to regulate and maintain the barrier function of endothelium. Monolayer integrity is controlled by the vascular endothelial–cadherin (VE–cadherin) complex, which mediates homotypic binding between cells and is the central component of endothelial cell–cell junctions (adherens junctions).8–10 This complex consists of the transmembrane protein VE–cadherin and intracellularly associated catenins and adaptor proteins that link VE–cadherin to the F-actin cytoskeleton.10–12 Adhesion of endothelial cells to the vascular ECM occurs through various transmembrane adhesion receptors, in particular via integrins.13,14 Integrins sense the composition and rigidity of the ECM. Their interaction with ECM components triggers the

See cover image
recruitment of adaptor and signaling molecules to integrin cytoplasmic tails. This provides them, in analogy to the organization of cadherin complexes, with a structural and functional link to the F-actin cytoskeleton.\textsuperscript{15–17}

Importantly, the phenotype of endothelium, which depends on cell adhesion, is different across the vascular network, and its morphology strongly reflects local endothelial function.\textsuperscript{18} Microscopic imaging of distinct vascular beds from mice reveals that endothelial cell–cell junctions gradually change organization during embryonic development\textsuperscript{19} and rapidly remodel during inflammation\textsuperscript{20} or angiogenesis.\textsuperscript{21} In bovine or mouse aorta, endothelial cells contain F-actin fibers, which are thought to depend on high shear flow rates of the bloodstream.\textsuperscript{22,23} The polymerization of F-actin is important to change junctional and cytoskeletal structures required for endothelial cell alignment in the direction of flow.\textsuperscript{24} Moreover, inflammatory stimuli induce F-actin fiber formation in rat endothelial cells within the vascular wall in situ.\textsuperscript{25} It is not yet known to what structures F-actin fibers attach in the endothelium of blood vessels, and it is also unclear if organization of the actin cytoskeleton relates to differences in endothelium of human arteries and veins.

In cultured cells on 2D ECM substrates, integrin-based adhesions manifest in so-called focal adhesions (FAs) at the end of F-actin bundles.\textsuperscript{26,27} Integrins are essential for vascular

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>FA</td>
<td>focal adhesion</td>
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<td>VE-cadherin</td>
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**Figure 1.** En face imaging of an umbilical vein. Different single channel and merged confocal images from a z-stack of the endothelial monolayer and subendothelial cellular layers of an umbilical vein immunofluorescently stained for vascular endothelial (VE)–cadherin (red), F-actin (green), and Hoechst (blue). See Movie 1 in the online-only Data Supplement for the complete z-stack of this stained umbilical vein. Scale bar 20 μm.

**Figure 2.** En face imaging of the endothelium of a human mesenteric artery. **A**, Procedure of preparation of human vessels for en face immunofluorescence microscopy. Images 1 to 5 is a photo series of isolation of a mesenteric artery: section of obtained mesentery (1), removal of fatty tissue (2), the isolated artery (3), immobilization of the artery with pins on a silicone layer and longitudinal opening (4), and fixation and antibody stainings from the luminal side of the vessel (5). Photo 6 is a confocal image of an immunofluorescence staining of mesenteric arterial endothelium stained for vascular endothelial (VE)–cadherin (red) and F-actin (green). Scale bar 20 μm. **B**, Single channel and merged confocal images of enlarged views from regions of interest from image in A6 that show different adherens junction conformations in mesenteric arteries: linear adherens junctions, focal adherens junctions, and other adherens junctions. White arrows indicate perpendicular F-actin bundles connected to focal adherens junctions. Scale bar 5 μm.
development and for adhesion of endothelial cells to ECM in the vascular wall. However, evidence for local regulation of endothelial integrin–based FAs by F-actin in the vasculature is limited. FAs in vivo have been described in basal epidermal cells in the skin and in tubular cells of injured kidneys of rodents. Also analysis of vascular tissue sections shows that integrins localize to the basal part of endothelial cells. However, integrin-based adhesion structures of cells in 3D matrices (ie, mesenchymal cells or tumor cells) function differently compared with cells in 2D conditions. The existence and the role of F-actin–anchored FAs in vivo, including in the endothelium, is therefore still debated today. Here, we used an ex vivo protocol for en face multicolor immunofluorescence imaging of human arterial and venous blood vessels. With this approach, we demonstrate that arterial F-actin fibers are attached to integrin-based FAs and to VE–cadherin-based focal adherens junctions. The prominent F-actin fibers clearly distinguish the endothelial phenotypes of adult arteries from veins.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
En Face Microscopy of Human Umbilical Veins to Visualize the Endothelium at Subcellular Resolution
To study the morphology of endothelial cells within human vasculature, we first optimized immunofluorescence on isolated veins from human umbilical cord tissue that is commonly used as source for isolation of human umbilical vein endothelial cells. After isolation of veins from the umbilical cord, vessels were cannulated and flushed with saline to remove residual blood. After fixation, the veins were opened in the longitudinal direction to enable immunofluorescence staining and imaging of the endothelial monolayer en face (from the luminal side). Confocal microscopy of umbilical veins stained for VE–cadherin, F-actin, and nuclei revealed that this approach nicely visualizes at high resolution the cellular morphology of a human vascular endothelial monolayer (Figure 1). The endothelium, the only vascular cell type that stains positive for VE–cadherin, forms a tight monolayer and VE–cadherin localizes clearly at the cell–cell junctions between cells. The cell–cell junctions are aligned by circumferential F-actin cables in a manner that is similar to the organization of VE–cadherin and F-actin in stable junctions of in vitro cultured human umbilical vein endothelial cells, but not VE–cadherin, is also clearly present in the cell layers on the basal side of the endothelium that contain the vascular smooth muscle cells (Figure 1 and Movie I in the online-only Data Supplement). This result indicates that en face microscopy of immunostained umbilical veins visualizes the endothelial morphology within human vasculature at high resolution.

VE–Cadherin and F-Actin Organization in Human Mesenteric Arteries
To investigate the morphology of endothelium within human adult vasculature, we applied immunofluorescence on vessels that were isolated from healthy sections of the mesentery that remain after pathological analysis of colon resections from patients with cancer (Figure 2A, images 1–5). En face confocal microscopy shows that endothelial cells in human mesenteric arteries are connected by VE–cadherin-based cell–cell junctions as expected and, in addition, display prominent F-actin fibers that locate centrally in individual endothelial cells (Figure 2A, image 6). In cultured endothelial cells, adherens junctions continuously switch between stable and remodeling conformations depending on local F-actin dynamics. Stable cell–cell junctions are visible as linear contacts between cells paralleled by thick F-actin cables, which we clearly observe in endothelium of mesenteric arteries (Figure 2B, upper image row and Figure 3A). In addition, we find punctate endothelial

Figure 3. Central F-actin fibers in human adult arteries, but not in veins. Confocal images of immunofluorescent vascular endothelial (VE–cadherin (red) and F-actin (green) staining in human arteries and veins from mesentery (A), mammary tissue (B), epigastric tissue (C), or umbilical cord (D). Scale bars 20 μm.
adherens junctions that are connected to perpendicular F-actin bundles in mesenteric arteries (Figure 2B, middle image row), which are reminiscent of remodeling focal adherens junctions in endothelial cell cultures and dynamic endothelial junctions in the vasculature of mice. This indicates that F-actin regulates endothelial cell–cell junctions both in vitro and in vivo through a common mechanism. In addition, we find irregular cell–cell junctions in mesenteric arteries that seem not related to the organization of F-actin at first glance. However, these other adherens junctions do associate with thin F-actin bundles that are much less intense than the central F-actin fibers (Figure 2B, lower image row). Although not unexpected, these results further show that the organization of F-actin in mesenteric arteries is different from its organization in umbilical veins.

Comparison of Endothelial Phenotypes Between Human Arteries and Veins From Various Tissues
To further characterize human endothelia, we compared VE-cadherin and F-actin stainings in arteries and veins isolated from various human tissues. We first compared arteries and veins from the adult mesentery. Additionally, we studied isolated arteries and veins either from the umbilical cord or from healthy tissue of patients undergoing breast reconstruction. Confocal microscopy showed that in all analyzed vessels from the different tissue origins, the endothelium forms

**Figure 4.** Endothelial focal adhesions (FAs) in human mesenteric artery. Merged confocal images of immunofluorescence staining for phosphorylated pY118 paxillin (red), F-actin (green), and vascular endothelial (VE)–cadherin (blue) in cultured arterial endothelial cells (A) and in a human mesenteric artery (B). Scale bar 20 μm. C, Single channel and merged images of 2 enlargement views of FAs from the mesenteric artery in 4B. Scale bar 10 μm [in region of interest (ROI) 1] and 5 μm (in ROI 2). D, Single channel and merged images of an enlargement view of 4B (ROI 3) showing F-actin fibers ending at phosphopaxillin-positive FAs (indicated by white arrows) or VE–cadherin-positive focal adherens junctions (FAJs; indicated by yellow arrows). Scale bar 5 μm.
a tight monolayer and VE–cadherin demarcates the cell–cell junctions (Figure 3). Furthermore, immunofluorescence stainings without primary antibodies show that the detected VE–cadherin signal in endothelium of human vessels is specific (Figure 1 in the online-only Data Supplement). Interestingly, the endothelium of mesenteric arteries is different from mesenteric veins, as we observe no central F-actin cables in veins (Figure 3A). The same phenotypic distinction in F-actin organization was observed between arteries and veins from mammary (thoracic) or epigastric tissue (Figure 3B and 3C). We observed no clear differences between endothelial phenotypes in umbilical arteries and umbilical veins, both of which lack prominent central F-actin cables (Figure 3D). Within veins the endothelium is less thick than in arteries, and often the most intense F-actin signal does not follow the shape of endothelial cells. Instead, the F-actin signal originates from the underlying vascular smooth muscle cells (see also confocal z-stack in Figure 1 and Movie I in the online-only Data Supplement). These results indicate that the presence of central F-actin fibers distinguishes the endothelial phenotype of human adult arteries from veins.

**Visualization of Endothelial FAs in Human Mesenteric Arteries**

In vitro most F-actin fibers are anchored to adhesion protein structures. A small portion of the F-actin fibers we observe in endothelium of adult arteries are indeed connected to VE–cadherin-based cell-cell adhesions (Figure 2B), whereas most other F-actin bundles do not. F-actin fibers in cultured arterial endothelial cells are attached to integrin-based FAs that are marked by phosphorylated paxillin (pY118) and connect to the ECM (Figure 4A). To investigate if endothelial F-actin fibers in arteries are part of integrin-based adhesions, we stained mesenteric arteries for paxillin pY118, F-actin, and VE–cadherin. Confocal imaging showed that phosphorylated paxillin is distributed at the basal plane of endothelial cells in a dotted pattern indicative of FAs (Figure 4B). Detailed image analysis of subcellular regions show a clear colocalization of phosphorylated paxillin precisely at the ends of F-actin fibers (Figure 4C, ROI 1 and 2; colocalization is yellow). Also, the orientation of individual F-actin fibers is similar to the direction of the elongated shape of its anchored FA, indicating that they are part of the same cellular structure. At subcellular regions in the proximity of cell–cell junctions, we find that F-actin fibers can terminate in paxillin-positive FAs (Figure 4D, white arrows), whereas others end at VE–cadherin-based focal adhesions junctions that are devoid of paxillin (Figure 4D, yellow arrows). The existence of integrin-based adhesions in human arteries was confirmed by immunofluorescence imaging of phosphorylated focal adhesion kinase pY397 (Figure 5A) and by stainings with a paxillin antibody that recognizes the protein irrespective of its phosphorylation status (Figure 5B).

Next, we investigated if the structure of arterial ECM relates to endothelial F-actin fibers. Immunofluorescence stainings in adult arteries for the ECM component fibronectin clearly show that endothelial cells follow the orientation of deposited

**Figure 5.** Molecular analysis of endothelial focal adhesions and their association with extracellular matrix in arteries. Single channel and merged confocal images of immunofluorescence stainings for phosphorylated pY397 FAK (A) or paxillin (B) in red, F-actin (green) and vascular endothelial (VE)–cadherin (blue) in mesenteric arteries. Right image rows show zoomed images of focal adhesions. Scale bars 10 or 5 μm. C, Single channel and merged confocal images of immunofluorescence stainings for fibronectin (red), F-actin (green), or VE–cadherin (blue) in a mammary artery (of note, this artery was obtained from tissue of a patient who previously received radiation therapy). White arrows in the enlarged image row (right) show F-actin fibers that follow the orientation of fibronectin fibrils. Scale bars 30 or 5 μm. ROI indicates region of interest.
fibronectin (Figure 5C). Importantly, prominent endothelial F-actin fibers are aligned by fibronectin fibrils in the ECM, suggesting that vascular ECM and endothelial morphology are tightly correlated. Taken together, these results demonstrate the existence of endothelial FAs in human arterial endothelium.

**Biomechanical Properties of ECM Determine Endothelial Phenotype and FA Formation**

What causes the differences in endothelial morphology between arteries and veins and between adult and umbilical arteries? In part, differences between arteries and veins are governed by genetic factors. Nevertheless, also the biomechanical properties of blood vessels are critical components that affect endothelial function. Shear forces derived from blood flow are lower in veins compared with arteries. Because shear stress remodels FAs and promotes integrin activation in vitro, we first examined whether differences in laminar flow rates of arteries and veins could underlie the formation of endothelial F-actin fibers and FA in vessels. Both arterial and endothelial cells cultured in vitro aligned to laminar shear flow at 2 dynes/cm² (a venous flow speed) after 18 hours, displaying aligned F-actin fibers and FAs (Figure 6). Also laminar shear flow at an arterial speed of 15 dynes/cm² induced similar aligned phenotypes in both endothelial cell types (Figure 6). These results suggest that the difference between arterial and venous flow rates does not directly induce the arterial or venous endothelial phenotypes that are observed in human vessels.

Arteries and veins not only differ in blood flow rates, but veins are also more compliant (less stiff) than arteries, a property that is largely determined by the vascular ECM and activity of smooth muscle cells. The vascular wall of arteries...
and veins in the umbilical cord might be more elastic compared with adult vessels.\textsuperscript{50} Forces generated by stiff ECM are exerted on, and predominantly regulate, adhesions between cells and the ECM.\textsuperscript{51,52} The formation of FAs and organization of F-actin in cultured endothelial cells are promoted by substrate stiffening.\textsuperscript{53} This also applies to cultured arterial endothelial cells because we find that their morphology on soft matrices (0.5 kPa) reflects a venous phenotype (Figure 7A). By contrast, significantly increased number of F-actin–linked FAs form once the cells are cultured on top of stiffer matrices (25 kPa, or on glass; Figure 7A and 7B). Importantly, once human umbilical vein endothelial cells are cultured on 25-kPa stiff substrates or on glass, they also adopt an arterial endothelial phenotype and contain significantly more FAs compared to on soft 0.5-kPa substrates (Figure 7A and 7B). This suggests that endothelial morphology and function is in a large part determined by the biomechanical properties of its ECM environment rather than intrinsic differences between arterial and venous cells.

**Discussion**

This study presents results from a microscopy approach for detailed en face analysis of endothelial monolayers of ex vivo human vasculature. The 2 key conclusions from the experiments are (i) endothelial FAs anchored to F-actin fibers exist in adult human arteries and (ii) F-actin is differently organized in adult arteries and veins. We propose that the biomechanical properties of the vascular wall regulate this endothelial characteristic. Furthermore, we observed different conformations of VE-cadherin-based cell–cell junctions that may relate to the distribution of F-actin at or near the junctions.

**Distinct Endothelial Properties in Arteries and Veins**

As mentioned earlier, the organization and the function of endothelial monolayers depends on location within the vascular network.\textsuperscript{18} Interestingly, perturbations of the endothelial barrier induced by inflammatory agents occur preferentially at the venous side of the circulation.\textsuperscript{54,55} However, much of our knowledge concerning morphological and functional responses of endothelial monolayers is based on in vitro studies. In particular, vascular permeability factors activate signaling pathways that transiently remodel F-actin stress fibers and actomyosin contractility to induce endothelial permeability.\textsuperscript{56-60} Such induced alterations in the F-actin cytoskeleton depend on cell-ECM adhesion and directly influence VE-cadherin function and cell–cell junction adhesiveness.\textsuperscript{54,61-63} Importantly, 2 recent studies showed that phosphorylation of VE-cadherin at tyrosine 685 is specifically high in venous vasculature of mice, but not in arteries.\textsuperscript{64,65} Inflammatory agents promote the phosphorylation of VE-cadherin at Y685, which correlates with and is required for vascular leak formation in venules.\textsuperscript{54} These results strongly indicate that the endothelium of arteries and veins contain intrinsic differences that might explain their distinctive susceptibility to transient vascular leaks during inflammation. Although not shown, it is tempting to speculate that the differences we observe in endothelium of human arteries and veins somehow contribute to this susceptibility.

**Elucidating Abnormalities in Blood Vessels That Underlie Vascular Disease**

To elucidate the mechanism that underlies chronic endothelial permeability in cardiovascular disease, it would be interesting to analyze age-related ECM stiffening and consequent regulation of F-actin–anchored FAs in human arteries. Gradual changes in the composition of the vascular ECM are the major cause for arterial wall stiffening during aging. Flexible components of the ECM, mainly elastin, are degraded, whereas deposition of more rigid components, such as collagen, is increased. This, combined with increased cross-linking to other matrix proteins including fibronectin, enhances the rigidity of the vessel wall.\textsuperscript{4,5} Atomic force microscopy indentation measurements on the subendothelial matrix of mouse thoracic aortas shows that rigidity is increased 2- to 3-fold in aged mice.\textsuperscript{5} Such elevation in stiffness is sufficient to increase endothelial permeability in aging arteries and is suggested to drive atherosclerosis and other cardiovascular diseases.\textsuperscript{5,61,66} Determining the endothelial phenotype in arteries from young and old individuals, or in vessels from healthy or cardiovascular disease tissue, combined with subendothelial mechanical indentation measurements of the vascular wall, would demonstrate whether age-related ECM stiffening is associated with regulation of endothelial adhesions. Improving our fundamental knowledge of the role of endothelial adhesion in pathogenesis of cardiovascular disease may eventually be used for therapeutic intervention. Such intervening strategies should be focused on the primary response of endothelial cells to increased arterial ECM stiffening.

Taken together, this study shows endothelial adhesion structures in human vessels at a resolution that was not shown before. The identified FAs anchored to F-actin are a key feature of adult arteries, but not of veins or vasculature of the umbilical cord. At this point, it is still unclear what endothelial function is related to the observed differences. However, we suggest that these differences in endothelial phenotype can be attributed to the biomechanical properties of the surrounding vessel wall. Finally, we recommend en face microscopy for molecular assessment of human endothelium to understand endothelial dysfunction in the development of vascular disease.

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Disclosures

None.

References

Endothelial cell adhesion is essential for barrier function, angiogenesis, and inflammation. The phenotype of endothelium, which depends on cell adhesion, is different across the vascular network, and its morphology strongly reflects local endothelial function. Changes in the extracellular matrix of the vascular wall are transmitted through these adhesion structures, and when such changes are prolonged, they cause permanent perturbation of endothelial functions, as occurs during age-related cardiovascular disease or chronic inflammation. It is therefore important to understand the regulation of cell adhesion in vascular health and disease. This study shows in detail how endothelial adhesion structures are organized in healthy human blood vessels and, importantly, differ between adult arteries and veins.

Significance
F-Actin–Anchored Focal Adhesions Distinguish Endothelial Phenotypes of Human Arteries and Veins

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Supplemental Figure I: Investigating specificity of VE-cadherin immuno-stainings in human vessels. Confocal images of immunofluorescent VE-cadherin (red) Hoechst (blue) or secondary only stainings in human epigastric arteries. Scale bars 10 μm.
LEGEND FOR SUPPLEMENTAL VIDEO FILE

**Supplemental Movie 1.** Confocal z-stack of an umbilical vein immunofluorescently stained for VE-cadherin (red), F-actin (green) and Hoechst (blue) from Fig. 1. Z-resolution is 0.47 μm per stack.
MATERIAL & METHODS

Human vessel preparations for microscopy

Umbilical cords were obtained from the Gynecology and Obstetrics unit at the Slotervaart Hospital (Amsterdam, the Netherlands), stored in phosphate buffered saline supplemented with 1 mM calcium chloride and 0.5 mM magnesium chloride (PBS++) at 4°C and processed within 24h after birth. The umbilical vein was cannulated and flushed with warm PBS++ to remove residual blood. Thereafter, the umbilical cord was cut in sections of approximately 1 cm in length. The umbilical vein was opened in the longitudinal direction and residual connective tissue was carefully removed, without damaging the umbilical arteries. Subsequently, the arteries were prepared similarly. After washing with PBS++, the opened umbilical vasculature was fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). The fixed umbilical veins and arteries were stored in PBS++ at 4°C before en face immunofluorescent stainings.

Healthy human blood vessels were obtained from tissue that remains after pathological analysis of mesentery from patients that underwent intestinal tumor resection in the Academic Medical Center (Amsterdam, the Netherlands). Mammary and epigastric vessels were isolated from remaining tissue of patients that underwent breast reconstruction in the Antoni van Leeuwenhoek Hospital (Amsterdam, the Netherlands). All vessels were obtained with informed consent and according the (Dutch) guidelines for secondary used materials. The vessels (ranging from 0.5 – 2.5 cm in length) were stored in PBS++ at 4°C for no longer than 24 hours until further preparation. The surrounding fatty tissue was removed and vessels were immobilized with small pins on a silicone layer within a petri dish to cut open the vessel in the longitudinal direction. Thereafter, the vessels were carefully washed with PBS++ to remove residual (clotted) blood and fixed with 4% PFA for 10 minutes at RT.

Cell culture

Primary human arterial endothelial cells (HAECs) from different donors and pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Baltimore M.D.) and cultured in EGM-2 medium supplemented with EGM-2 bulletkit (Lonza). Cells were passaged by trypsinization. Passage 2 – 4 HAECs or HUVECs were plated on fibronectin-coated glass coverslips, ibi-Treat μ-Slide VI°4 flow slides (ibidi) or on Softview glass-bottom dishes coated with polyacrylamide crosslinked with different bisacrylamide concentrated hydrogels of 0.5 or
25 kPa rigidity (Matrigen, Brea, CA). After overnight incubation confluent monolayers of HAECs or HUVECs were fixed with 4% PFA/PBS** for 10 minutes at RT prior to immunofluorescence stainings. For flow experiments µ-Slides were connected to a Watson-Marlow 520Du pump using a flow-through-kit from ibidi and cells (4 hours after plating) were subjected to laminar shear flow at venous (2 dyne/cm$^2$) or arterial (15 dyne/cm$^2$) rates for 18 hours with supplemented EGM-2 medium.

**Antibodies**

Mouse monoclonal antibodies against VE-cadherin, clone F-8 (Santa Cruz Biotechnology) or clone 55-7H1 directly coupled to Alexa Fluor 647 (BD Pharmingen), were used in combination with rabbit polyclonal antibodies raised against phosphorylated paxillin (pY118) and phosphorylated focal adhesion kinase (FAK, pY397) (Invitrogen). Rabbit polyclonal VE-cadherin antibody (Cayman) was used combined with mouse monoclonal clone 165 for paxillin and mouse monoclonal antibody against fibronectin, clone 10 (both from BD Transduction Laboratories). Secondary goat-anti-mouse or goat-anti-rabbit antibody coupled to Alexa Fluor 488, and goat-anti-mouse or goat-anti-rabbit secondary coupled to Alexa Fluor 568 were obtained from Invitrogen. We used phalloidin conjugate labeled with the fluorescent dye CF633 (Biotium) or Acti-stain 555 (Cytoskeleton, Inc.) to visualize F-actin. Hoechst 33342 (Molecular Probes) was used to stain the cell nuclei.

**Immunofluorescence microscopy**

Sections of fixed blood vessels from mesenteric, mammary, epigastric and umbilical cord tissue were immobilized on a petri dish coated with a silicone layer, with the endothelial side facing up, to perform en face immunofluorescent stainings. The vessels were permeabilized with 0.5% Triton X100 in PBS** for 10 minutes and blocked for 30 minutes with 2% bovine serum albumin (BSA) in PBS**. Next, the vessels were incubated for 1h at RT with primary antibodies, which were diluted in 0.5% BSA in PBS**. After washing with 0.5% BSA in PBS**, the vessels were incubated with secondary antibodies, including phalloidin and Hoechst diluted in 0.5% BSA in PBS++. Finally, the vessel was mounted with mowiol in a glass bottom microwell dish (MatTek Corporation) with the endothelium facing down for confocal laser scanning microscopy (Zeiss LSM 510) equipped with a 63x 1.4 N.A. Plan Apo oil objective and Argon, DioDe 405, HeNe 633 lasers. HAECs and HUVECs on glass coverslips or flexible substrates were immunofluorescently
stained using the same protocol and subsequently imaged in PBS on an upright widefield microscope (Zeiss Imager.Z2) equipped with a 40x 1.0 N.A. Pln Apo water objective and Hamamatsu Orca-R2 digital camera. HAECs and HUVECs on ibidi flow chambers were imaged in PBS on an inverted widefield microscope (Zeiss Observer.Z1) equipped with a 40x 1.4 N.A. Pln Apo oil objective and Hamamatsu Orca-R2 digital camera. All obtained images were enhanced for display with an unsharp mask filter and adjusted for brightness/contrast in ImageJ (National Insitutes of Health).

Quantifications and statistical analysis

In Figure 7 the number of FAs per cell was quantified using the cell counter plug-in in Image J software. For each experimental condition (HUVEC or HAEC plated on 0.5 kPa, 25 kPa, or glass substrates) at least 10 representative immunofluorescence images of two independent experiments were analyzed. For each picture, the number of phosphorylated paxillin pY118 puncta associated with F-actin were counted. Out-of-focus cells were excluded from the analysis. The ratio of the number of F-actin-connected FAs and the number of cells was determined. The values in the graph of Figure 7b are presented as average ± standard deviation. Statistical analysis and p-values were calculated using two-tailed Student’s t-tests and were considered significant at p-values < 0.01.