Chapter

Fluorescence Methods for the Analysis of Microtubule/Microfilament Involvement in the Regulation of Endothelial Barrier Function

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Abstract

The endothelial monolayer is located on the inner surface of blood vessels and provides an important barrier function controlling the transport of metabolites and nutrients through the vessel wall, both toward the circulating blood and in the direction of the underlying tissues. This function is provided by cytoskeletal structures that generate contractile and tensile forces, existing in equilibrium in the intact endothelium. In the case of cytoskeleton rearrangements, there are changes in the shape of cells and the formation of intercellular spaces, which lead to endothelial dysfunction. Deep understanding of endothelial barrier function maintaining is a crucial problem because this phenomenon is common for a number of pathological states and diseases (inflammation, asthma, sepsis, acute lung injury, diabetes, etc.) and can lead to severe organ dysfunction, as well as a complication upon the treatment by a number of anticancer pharmacological drugs. Microtubules and the actin cytoskeleton function in cooperation in normal endothelium and under conditions of the barrier loss. In this review, we describe the application of modern fluorescence methods for investigation and analysis of the individual characteristics of cytoskeletal elements whose reorganization affects endothelial permeability, to emphasize the role of microtubules/microfilament crosstalk in EC barrier regulation.

Keywords: fluorescence analysis, fluorescent microscopy, confocal laser scanning microscopy, structural illumination microscopy, endothelium, endothelial cells, endothelial barrier function, endothelial cytoskeleton, microtubules, microfilaments

1. Introduction

Endothelium—cell monolayer locolazing on the inner vascular surface is formed by specialized cells: endothelial cells (EC) or endotheliocytes. Endothelium functions as a selective permeable barrier between vascular blood flow and tissue fluid in organs comprising these vessels. It is involved in the regulation of

macromolecular transport and blood cell movement through the vessel wall. The integrity of vascular endothelium in any organ is critical for its functioning. Thus, corneal endothelium ensures its normal permeability and exchange with the connective stroma [1]. Vascular endothelium in lungs regulates the movement of fluids, macromolecules, and leukocytes into alveolar interstitial and air spaces [2, 3].

Lung endothelium lining the vessels (aorta, arteria, and others) are in close contact with each other, rendering the vascular wall into a tight barrier, which control such diverse processes as vascular tone, homeostasis, adhesion of platelets, and leukocytes to the vascular wall and permeability of vascular wall for cells and fluids [4, 5]. The integrity of the pulmonary EC monolayer, therefore, may be a critical requirement for the preservation of pulmonary function. This barrier is dynamic and highly prone to the regulation, by various stimuli, of physiological and pathological origin. Any breach within the EC barrier ends up in leakage of fluid from the lumen of the vessels into the plant tissue and/or alveolar lumen, severely impairing gas exchange. Disruption of endothelial barrier occurs during disease states like acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), which remains a significant explanation for morbidity and mortality with an overall fatality rate of 30–40% [6], results in the uncontrolled movement of fluid and macromolecules into the interstitium and pulmonary air spaces causing pulmonary edema [7]. Literature and our own data have proved that normal functioning of the endothelial barrier is provided by the balance between contracting and stretching forces generated by EC cytoskeleton [4, 8, 9] and cell adhesive structures are involved in their relationship [9–11].

In order to understand how such a complicated and multi-regulated system works, one should characterize its structure as a whole system (as well as each component separately) in normal conditions and study the complex changes that occur during barrier dysfunction and also investigate their reorganization in dynamics. The main focus of this chapter is fluorescence methods using for investigation of EC, including living endotheliocytes, and we will demonstrate what results have been achieved due to their applying.

2. Endoteliocyte cytoskeleton: interaction of components performing barrier function

Cellular cytoskeleton is a complex of fibrillar components that structurally and functionally ensures the normal course of numerous vital functions of the cell. The cytoskeleton provides the maintenance of the cell shape and all its changes during the life cycle, as well as the native architecture and intracellular organization of its organelles. The components of the cytoskeleton are involved in transport processes during the interphase and they guarantee successive mitotic phases passing in the process of cell division. Endothelial cell cytoskeleton, similarly to the cytoskeletons of the other cell types, consists of three types of filaments—microtubules, actin filaments (microfilaments), and intermediate filaments.

The components of the cytoskeleton are composed of various proteins. Two types of them—microtubules and microfilaments—are monoprotein structures: microtubule walls are polymerized from alpha- and beta-tubulin heterodimers, and microfilaments are built from actin in the cells of a wide variety of different tissues. Unlike tubulin-polymerized microtubules and actin filaments intermediate filaments do not have a highly conservative protein structure. Intermediate filaments consist of different proteins, their composition in the cell depends on the tissue type in which they are located. Intermediate filaments have a different protein composition not only in different tissues but also at different stages of differentiation as well

as at different stages of embryonic development. Intermediate filaments consisting of different proteins are expressed in different cell types. Moreover, in some types of cells, intermediate filaments consisting of different proteins can be present simultaneously.

Microtubules are an obligatory component of the cytoskeleton of all cells, they are highly dynamic polar biopolymer structures. As all other cytoskeleton components microtubules are multifunctional structures involved in various cellular processes, including directed cytoplasmic transport of vesicles and signaling molecules and changes in the cell shape during mitotic division as well as in interphase (during spreading, polarization, or movement).

Like the microtubules, actin filaments are liable for changes in cell shape and mobility. In contrast the intermediate filaments are mainly responsible for cell shape. Reorganization of the endotheliocyte cytoskeleton consisting of actin filaments, microtubules, and intermediate filaments and also the subsequent change within the cell shape creates a structural basis for the increase in vascular permeability. Historically, the contribution of various components of the cytoskeleton to the change in the shape of endotheliocytes during the development of barrier function has been studied in an unequal measure. In addition, the role of intermediate filaments is still poorly understood. The apparent role of actin filaments has been studied rather actively [2, 3, 12], whereas the involvement of the microtubule system during this process was revealed significantly later [13, 14]. The main achievement of recent years is that the understanding that each one component of the cell cytoskeleton is functioning with interrelations and their interactions are coordinated on different levels of the regulation. Moreover, cytoskeletal components are more or less interacting with adhesive structures of the cell; this complicated and finely regulated interaction allows the cells to implement the great variety of cellular, tissue, and organ functions. Within the present review, we shall analyze interactions of the cytoskeletal elements during changes in the endothelial permeability and emphasize the role of dynamic microtubules in the regulation of the barrier function of the endothelium.

As mentioned previously, intermediate filaments consisting of different proteins are expressed in different cell types. The homology of intermediate filament proteins is sometimes no more than 20%; nevertheless, based on biochemical, immunological, and structural similarities, five different types of intermediate filaments are distinguished: keratins (there are two types of proteins—type I (acidic keratins) and type II (basic keratins) —for the assembly of keratin intermediate filaments, proteins of both types are required—they form heteropolymers). Type III intermediate filament proteins include four proteins: desmin, vimentin, peripherin, and glial acidic protein. Each of these four proteins can form homopolymers, but besides that they are also able to form heteropolymers with other type III. Type IV intermediate filament proteins are expressed mainly in nerve cells: they include α-internexin and a triplet of neurofilament proteins: NF-L, NF-M, NF-H (neurofilament light, medium, and heavy proteins). The protein nestin, first discovered in the precursors of nerve cells, is sometimes referred to as a special type of intermediate filaments proteins; however, based on its structural features, nestin can be classified as type IV. Type V intermediate filament proteins include nuclear lamins. Vimentin filaments are the most abundant intermediate filaments of endothelial cells, and they are the least investigated, not only in endotheliocytes but also in other types of cells [15, 16]. Vimentin filaments are relatively stable structures, and their network remains almost unchanged in vitro in the process of monolayer formation [16]. Presumably, vimentin filaments might be involved in cytoskeletal interactions mediately: they might be crucial for centrosome positioning [17] and thus influence the microtubule system architecture. It seems that actin and vimentin filaments can

interact structurally because they are connected to the biochemical level [18, 19]. Thus, the tail domain of vimentin is shown to interact directly with actin filaments [19], but up to now, no vimentin binding domain of fibrillary actin has been found. Data on microtubules and intermediate filaments coordination are still desultory; likely, kinesin [20] and a few other proteins [21, 22] can act as adaptor proteins between vimentin and microtubules. This insufficient attention given to intermediate filaments will be easily explained because actin filaments are generally assumed the key player crucial for normal endothelial permeability.

Actin polymerization is extremely dynamic in the endothelial cells. This dynamicity allows actin structures to rapidly rearrange and alter the static phenotype characterized by a large cortical actin ring and a minimal number of stress fibers to the so-called activated phenotype characterized by a deficient layer of cortical actin (sometimes up to its complete absence) with numerous stress fibers compressing the cell. Moreover, just this explains why it was originally thought that the actin system should be the central cytoskeletal component responsible for the functioning of the endothelium and development of its dysfunction [4]. A vital role of actin filaments in barrier dysfunction development was shown in many works. Notably, treatment of cultured cells with cytochalasin D that disrupt the actin cytoskeleton induced a drastic increase in the endothelial monolayer permeability [23], whereas treatment with phalloidin inhibiting actin depolymerization prevented the looks of the barrier dysfunction [24]. The interaction of actin with many binding proteins (filamin, vinculin, etc.) and adhesive structures chargeable for the barrier function also seems to evidence its key role within the development of endothelial dysfunction [4].

As polymer actin microfilaments are highly dynamic structures, their exchange time at the cell edge is just a few seconds [25]; nevertheless, they effectively regulate the cell shape and under in vitro conditions retain a stretched cell state. Focal [26, 27] and intercellular contacts [13, 14, 28, 29] also are able to rapidly, during several minutes, assemble and disassemble. Actin filaments are actively involved in the interactions with adhesive structures of the cell [5, 30]. It is generally assumed that the intercellular gap formation is regulated just by the balance of competing compressing forces, which curtail the cell toward its center, and forces generated by adhesive intercellular junctions and focal cell-substrate adhesions jointly regulating the cell shape [31]. The two competing forces in this model are related through actin fibers, which interact with the various adhesive molecules of the focal adhesions and intercellular adherent junctions of membranes.

However, recent works have shown that actin filaments do not seem to be instantly involved in the manifestation of endothelial barrier dysfunction, while microtubules are just the structure liable for the primary stages of this process [9, 32]. Thus, thrombin, which is produced on the surface of damaged cells from prothrombin circulating in the blood and inducing blood coagulation, can disturb the barrier function of the endothelium both in vitro and in vivo. It was shown that thrombin theatment leads to increase the permeability of the endothelium that is associated with a rapid decrease in the number of microtubules on the cell periphery near the margin and the microtubule system reorganization in the inner cytoplasm of endothelial cell during the first minutes of exposure. Actin stress-fibers are formed step-by-step, and therefore the maximal effect is observed only 30 min after treatment with thrombin [9, 29]. Thus, the development of microtubule response was faster than the reorganization of the actin filament system liable for the subsequent changes in the shape of the cells during the development of barrier dysfunction. Permanent depolymerization of microtubules after nocodazole exposure also induced an increase in the permeability of the endothelial cell monolayer and triggered a molecular cascade resulting in barrier dysfunction [9, 33].

The increase in the endothelial barrier permeability under nocodazole treatment is directly associated with the degree of depolymerization of peripheral microtubules. Endothelial barrier permeability increase after nocodazole treatment is directly associated with the degree of peripheral microtubules depolymerization. Normal endothelial barrier permeability can be disturbed even on minimal disruption of peripheral microtubules when the system of actin filaments remains intact and no morphological changes are observed [9]. The contraction of the cell body within the absence of stress fibers is surprising, but not unique fact, as already described earlier [31]. In the great majority of cases, despite this fact, barrier dysfunction is accompanied by stress fibers formation not depending on factors responsible for disturbance of the endothelial permeability, either drugs or pharmacological preparations [13, 14, 34] or neurohumoral factors [13, 35]. Thus, the disruption of peripheral microtubules was shown to be necessary and sufficient for arising of endothelial barrier dysfunction [9, 32].

Microtubules, the primary target of recent investigation, are involved in the most active dynamic interaction with both actin filaments and adhesive structures of various types of cells. During recent decades, these interactions were actively studied. The interaction of microtubules with actin filaments was found to be important for functional activities of various cells [31, 36], including endotheliocytes [37, 38], and microtubules dynamic properties are especially important for such interactions [9, 39].

Microtubules are highly (but less then microfilaments) dynamic cytoskeleton structures. The ends of individual microtubule are continuously growing or shortening even in cases when no visual changes are observed in the cell cytoplasm. Microtubule ends are growing or disassembling at distances of several microns [40, 41], and the microtubule system is continuously exchanging with the cytoplasmic pool of dissolved tubulin with exchange time of 5–20 min [42, 43]. Behavior described was named "dynamic instability" [40, 41], because each individual microtubule, as discriminated from the whole system, is not in a stationary state. However, notwithstanding the dynamic behavior of individual microtubules, changes in the microtubule system organization occur rather slowly even in the cells adapted to the movement. At the same time in nonmotile or poorly motile cells the microtubule system is maintained virtually unchanged in cell space and time. The assembly of microtubules is characterized by attachment of newly polymerized fragments of microtubules with GTP-β-tubulin. Because β-tubulin gradually hydrolyzes its GTP upon incorporation into the microtubule, GDP-β-tubulin dominates in the remaining a part of microtubules. Plus-end proteins specifically bind to the GTP-enriched fragments of microtubules [44, 45]. Microtubule plus-end proteins represent an extensive and varied group, and the most specific role in microtubules polymerization belongs to end-binding (EB) proteins family. Two proteins from the family—EB1 and EB3—interact with tubulin directly. Structural proteins CLIP115 and CLIP 170 belong to the cytoplasmic linker proteins family, bind EB1 and EB3. These proteins alternately bind with structural proteins of the CLASP family (CLIP-associated proteins). Microtubule tips complex of plus-end proteins play a crucial role in regulation of intracellular traffics and are directly involved in the interaction of microtubules with the cell boundary and the structures localized in this region [45–47].

It is thought that just the dynamic instability of microtubule ends and their ability to change frequently the assembly/disassembly stages allows the microtubules to locally modulate the dynamics of cell contacts [26, 27, 48, 49] and also to manage the dynamics of intercellular junctions [27, 38, 50, 51]. Moreover, microtubules can control internal cell organization.

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3. Fluorescense methods using for investigation of endotheliocytes

3.1 Immunofluorescence microscopy

Traditionally, immunochemical labeling of proteins of interest and the intracellular structures formed by them is used for structural studies. After fixation and immunostaining, it is possible to investigate cells by widefield immunofluorescence microscopy. The method was well known and widely used from the middle of the last century; with its help, numerous studies, including the investigation of cell cytoskeleton components, were performed. Using immunofluorescence analysis, the authors showed that cytoplasmic actin in endotheliocytes is organized in structures of three types: membrane cytoskeleton, cortical ringlike structures, and stress fibers [31]. Using double immunofluorescent staining of endothelial cells, mutual arrangement of microtubules and actin stress fibers has been described (**Figure 1a**) as well as the positional relationship of microtubules and specific endothelial VE-cadherin cell-cell contacts (**Figure 1b**).

Active creation of new specific antibodies and synthesis of recent highly effective and resistant to fading fluorescent labels for their detection make the method widely used nowadays.

We present our protocol for double immunofluorescence labeling of cytoskeletal components (microtubules, actin filaments, and vimentin intermediate filaments) adapted for endothelial cells. To quantify the obtained data, we have developed methods for analyzing the fluorescence intensity of immunostained samples [11, 16], which are also given below.

3.1.1 Cell cultures

Two cell lines of human endothelium, hybrid EA.hy926 cells and HPAEC (endothelium of human lung artery), were used in the experiments. EA.hy926 cells were obtained by fusion of primary endotheliocytes isolated from human umbilical vein and thioguanine-resistant clone of A549 cells (human lung carcinoma). According to ATTC and the manufacturer's database, EA.hy926 cells that have undergone more than 100 division cycles manifest all the basic properties common for differentiated endothelium. The cells were grown in DMEM medium at 37°C and 5% CO2. EA.hy926 cells were used in experiments at the sixth to tenth passages. HPAEC cells (Clonetics BioWhittaker Inc., United States) were the primary culture

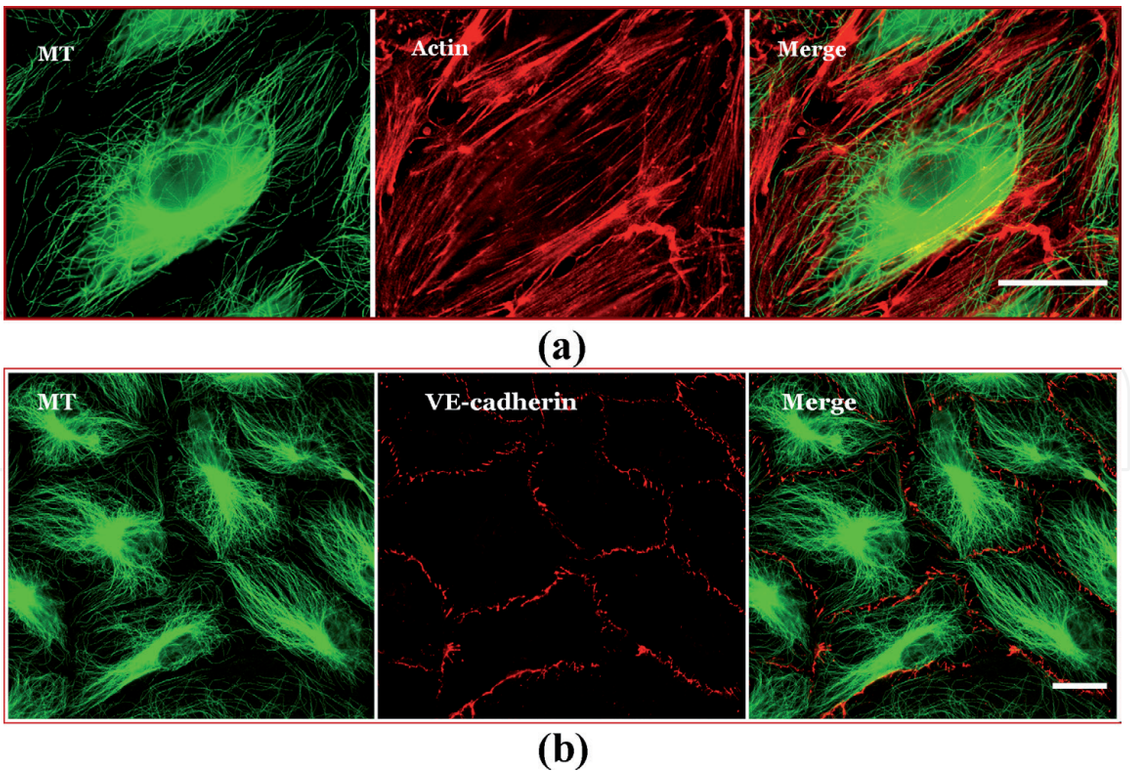

Figure 1.
(a) Double immunofluorescent staining of endothelial cells of human pulmonary artery with antibodies against β-tubulin (green) for detecting microtubules (MT) and with antibodies against actin (red) for detecting actin stress-fibers. (b) Double immunofluorescent staining of endothelial cells of the human pulmonary artery with antibodies against β-tubulin (green) and VE-cadherin (red). Stained preparations were analyzed under a Nikon Eclipse TE2000 microscope (Nikon Intech Co., Japan). Images were recorded using a 60x-objective with Hamamatsu ORCA-2 digital cooled P3C camera (Hamamatsu Photonics, Japan) with MetaView software (Universal Imaging, USA). Scale bar: 20 μm. (from [56]; with modification).

established from human pulmonary artery. The cells were maintained in EGM-2 medium (Clonetics BioWhittaker Inc., United States) at 37°C and 5% CO2.

3.1.2 Immunofluorescent labeling

Cells were fixed with three different methods: (1) for subsequent labeling by antibodies against β-tubulin and for dobble-labeling by antibodies against β-tubulin and β-actin, the cells were fixed with methanol (Sigma, United States) at −20°C and for 8 min. (2) For subsequent dobble-labeling by antibodies against vimentin and β-actin, the cells were fixed for 10 min at room temperature with formalin (Sigma) (4% solution in phosphate buffer solution [PBS], pH 6.8), washed thrice in PBS for 10 min, permeabilized for 15 min with Triton X-100 (Sigma) (0.1% solution in PBS). For fluorescence back ground degradation, the cells were treated with sodium borohydride NaBH4 (Sigma) (0.2% solution in PBS, 30 min) and washed thrice in PBS for 10 min prior to labeling with antibodies. For immunofluorescent staining, the samples were incubated with primary (30 min, at 37°C) and secondary antibodies (30 min, at 37°C). (3) Actin isoforms were visualized in cells fixed with 1% paraformaldehyde (Sigma, United States) in DMEM with HEPES buffer for 15 min, washed with PBS, and postfixed with methanol for 5 min. The same fixation procedure was used for dobble-labeling by antibodies against β-tubulin and γ-actin.

Vimentin intermediate filaments were labeled with antibodies to vimentin (BD Biosciences, United States, dilution 1:200); actin filaments, with antibodies against β-actin (Invitrogen, United States, dilution 1: 200); and microtubules, with monoclonal murine antibodies to β-tubulin (Covance, United States, dilution 1: 200).

Actin isoforms were visualized with mouse monoclonal antibodies to cytoplasmic β- and γ-actin isoforms (Dugina et al., 2009). Cells were washed with DMEM containing 20 mM HEPES at 37°C, fixed in 1% PFA in prewarmed (37°C) DMEM (30 min) and postfixed with methanol (–20°C) for 5 min. Cells were incubated with the following primary monoclonal antibodies: to β-actin (mAb 4C2, IgG1) and to γ-actin (mAb 2A3, IgG2b). Depending on the purpose of the study, as secondary antibodies, FITC- and TRITC-conjugated goat antimouse IgG1, TRITC-conjugated goat anti-mouse IgG2b (Southern Biotechnology, Associates, Birmingham, AL), or Alexa-Fluor-488-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen) were used in dilution 1:1000.

Coverslips were mounted on object glasses by using Mowiol as the embedding medium. To preserve samples, edges of coverslips were covered with nail polish.

Fluorescent mages were acquired on the confocal microscope LSM510 (Zeiss, Oberkochen, Germany) equipped with oil-immersion objectives (Plan-Neofluar 63 1.4 and PlanFluar 100 1.45, Zeiss). Single optical sections were scanned with ~1 μm thickness near the basal level of the cell. Stacks with z-step of 0.3–0.5 μm were collected as serial optical sections. Images were processed using Adobe Photoshop 7.0 (Adobe Inc., USA) software.

3.1.3 Imaging and image processing

Immunofluorescently stained endothelial monolayer semples were examined under a Nikon Eclipse TE2000 microscope (Nikon Intech Co., Japan) supplied with a 60/1.4 objective. We preliminary scanned of the samples, and the most spread cells were selected for the following analysis.

Images were collected with Hamamatsu ORCA-2 (Hamamatsu Photonics, Japan) digital cooled CCD camera supported with MetaView software (Universal Imaging, USA). The resolution of 12-bit digital images was 9 pixel/μm. Image processing was performed using MetaMorph (Universal Imaging, USA), ImageJ software (NIH), and Adobe Photoshop 7.0 (Adobe Inc., USA) softwares.

3.1.4 Quantitative assay of the actin filament system

Reorganization of actin cytoskeleton structure was analyzed by fluorescence intensity per unit area. At first, we chose a 10 μm^2 area on the captured images in the region of leading edge and contacts with neighboring cells. In this region, the average fluorescence intensity was measured using the ImageJ software. The chosen regions were not 5 μm from the cellular edge. For the quantitative analysis of actin filaments changes, the following parameters were used: relative area occupied by actin filaments, thickness of actin fibers, and length of actin fibers. To estimate the relative area on digital images, we used the following algorithm: the cell contour was outlined, then the cell area occupied by ImageJ (function "Polygon selection"), and the outlined areas were measured (function "Measure"). The ratio of the area occupied by actin filaments was calculated to the total cell area. The obtained data were exported into Excel for statistical analysis and for the construction of histograms. To measure the thickness of actin fibers on cell digital images in the ImageJ, the line was drawn perpendicular to the long fibril axis, and the line of the obtained line corresponded to the thickness of the actin fibers in the place of measurement.

3.1.5 Quantitative assay of the microtubule system

Changes in the microtubule system organization were assessed with the ImageJ software by counting the number of microtubule ends in the area of free cellular

lamella and in the zone of cell-cell (intercellular) contacts. The measurements were done in areas of 10 μm length and 2 μm width. Two approaches were used: (1) the microtubule number was counted directly if microtubules were well distinguished visually (at the lamella edge with a rather thin cytoplasm). (2) If the microtubule density prevented their precise identification, the microtubule number was counted with the ImageJ software by the peaks of fluorescence on digital images. Fluorescence peaks higher the background and corresponding to the microtubule position were registered on a section with 10 μm length and 2 μm width. Fluorescence peaks 25% or higher than the background value were taken for the assay of the microtubule number. Statistical treatment was done with the Sigma Plot 12.5 (SPSS Science, United States) and Excel (Microsoft Corp., United States) software. The significant difference between analyzed samples was assayed with the t-test (Student criterion) or nonparametric U-test (Wilcoxon-Mann-Whitney criterion).

3.2 Confocal laser scanning microscopy

The appearance of confocal laser scanning microscopy allowed researchers to get better quality images with high resolution in the plane of the analyzed sample, along the XY-axis.

Confocal laser scanning microscopy is a kind of light optical microscopy, which has significant contrast and spatial resolution compared to classical light microscopy, which is achieved by using a point aperture (pinhole) located in the image plane and restricting the flow of background scattered light emitted not from the focal plane of the lens [57]. This allows one to obtain a series of images at various depths of the focal plane inside the sample (the so-called optical sectioning of the sample by depth) and then reconstruct a three-dimensional image of the sample from these series.

In endothelial cells, actin cytoskeleton is represented by actin stress fibers [58] (**Figure 2**), cortical actin filament network, and membrane skeleton [31]. Confocal microscopy was the method that made it possible to characterize these structures and describe their mutual arrangement in the cell volume. The latter is located beneath the plasma membrane and reaches the width of up to several nanometers. It is composed of relatively short actin filaments, spectrin, ankyrin, and other proteins [31]. The membrane skeleton determines membrane architecture and provides membrane mechanical stability. It includes spectrin and spectrin-stabilizing proteins, such as F-actin, α-catenin, adducin. The cortical actin network consists of long actin filaments stabilized by actin-binding proteins (cortactin, filamin, spectrin, WASP, VASP, etc.) [31]. The membrane skeleton is mainly involved in maintaining cell shape, whereas the cortical network interacts with adjacent cells and extracellular matrix. Stress fibers containing short microfilaments with alternating polarity are found across the entire cell cytosol. Stress fibers are bundles of actin filament bound to α-actinin and other actin-binding proteins. In endothelial cells, actin stress fibers are mainly involved in enabling cell motility and contraction [31].

Similar to actins in other cell types, β- and γ-actin isoforms in endothelial cell can assemble in various intracellular structures. In human pulmonary artery endothelial cells (HPAECs) and human umbilical vein EA.hy926 cells, β-actin was found mostly in stress fibers [11, 16].

Staining with specific anti-β-actin antibodies detected cytosolic bundles of stress fibers and spheroid microparticles, whereas γ-actin formed branched cortical actin network. Moreover, the density of the γ-actin network was higher in EA.hy926 cells compared to HPAECs [16].

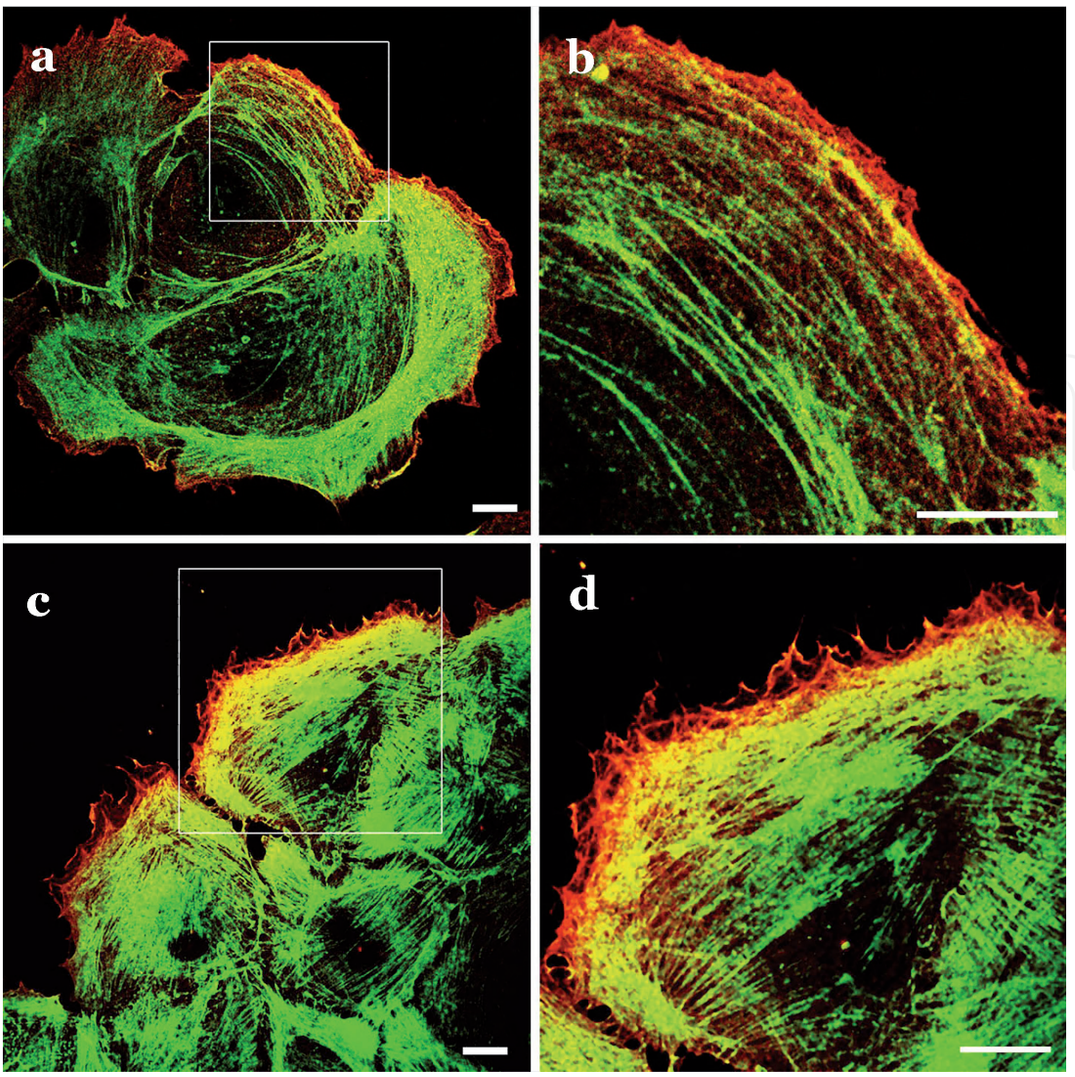

Figure 2.
Double immunofluorescence staining of β- and γ-actins in aorta endothelial cells in vitro. (a, b) In spread endothelial cells, β-actin forms circular bundles, whereas γ-actin forms lamellar microfilament meshwork. (b) Large magnification of boxed area on panel (a); (a) single optical x/y section across the basal cell level (scale bar, 10 μm). (c, d) β- and γ-actins in the leading edge of polarized endothelial cells moving into the experimental wound. Extensive γ-actin network is discovered in the leading edge protrusions of moving cells, whereas β-actin localizes in the microfilament bundles and in the area of cell-cell contacts. (d) Large magnification of boxed area on panel (c); (c) single optical x/y section across basal cell level. Scale bar, 10 μm. (from [58]; with modification).

For the analysis of β- and γ-actins in spread endothelial cells (**Figure 2**) immunofluorescent images were acquired using a confocal microscope (LSM510, Zeiss, Oberkochen, Germany) equipped with oil-immersion objectives (Plan-Neofluar 63 1.4 and PlanFluar 100 1.45, Zeiss). Single optical sections were scanned with ~1 μm thickness in the basal level of the cell. Stacks of serial optical sections (with z-step of 0.3–0.5 μm) were collected. Images were processed using Adobe Photoshop software.

Confocal microscopy as the method allowed reconstructing the position of the analyzed structures in the cell volume; however, the Z-axis resolution was insufficient, which stimulated the further development of microscopic technology.

3.3 Structural illumination microscopy

Structured illumination microscopy (SIM) is a relatively new high resolution method of microscopy analysis objects, which overcomes the shortcomings of

confocal laser scanning microscopy. The main goal that SIM solves as the modern light microscopy method is to enhance spatial resolution. The main innovation of the method is "patterned light"—for the observation of semples not direct, but so-called structural illumination is used. SIM method provides illuminating the sample with patterned light and using software to analyze the information in Moiré fringes outside the normal range of observation [59]. Reconstruction software deciphers the images at about 2-fold higher resolution than the diffraction limit, approximately equal to 100 nm. Significant advantages of SIM has over other microscopy methods are (1) SIM can be used for imaging thicker sections; (2) for 3D imaging; and (3) for live-cell imaging. Additionally, it is possible to increase the image quality with photostable and more bright and dyes as well as precise targeting. Fluorescent proteins are commonly used for SIM investigations of live cells in addition to multiplexing with organic dyes and Qdot® probes. SIM itself, as well as in combination with other methods of analysis (proximity ligation assay, etc.), allows not only to describe the relative position of the structures of interest, but also—due to their high resolution—to make conclusions about their colocalization.

Here, we represent the application of modern fluorescence methods for investigation and analysis of the individual characteristics and interrelation of dynamic cytoskeletal components—microtubules and actin microfilament in the regulation of endothelial permeability (**Figure 3**).

Microtubules were labeled with monoclonal murine antibodies to β-tubulin (Covance, United States), in dilution 1:200, actin filaments were labeled with antibodies to β-actin (Invitrogen, United States), in dilution 1:200. Alexa-Fluor-568-conjugated (ThermoFisher Sci) or Alexa-Fluor-488-conjugated (Molecular Probes, Invitrogen) secondary antibodies were used (both in dilution 1:1000).

For the samples, preparation cells were mounted in Mowiol 4-88 (Calbiochem). To prevent photobleaching, 1% DABCO solution was added to the mounting dye. Cells and examined on a Nikon N-SIM (Nikon) equipped with NA oil immersion objective 100x/1.49, 488 nm and 561 nm diode laser excitation. For image aqusition, stacks with z-steps of 0.12 μm were used. Image stacks were acquired with EMCCD camera (iXon 897, Andor, effective pixel size 60 nm), herewith, exposure conditions were adjusted to get typical yield about 5000 max counts (16-bit raw image) while keeping bleaching minimal. Serial optical sections of the same cell taken in widefield mode were deconvolved using the AutoQuant blind deconvolution algorithm. Image acquisition, SIM image reconstruction, and data alignment were preformed using NIS-Elements 4.2 software (Nikon).

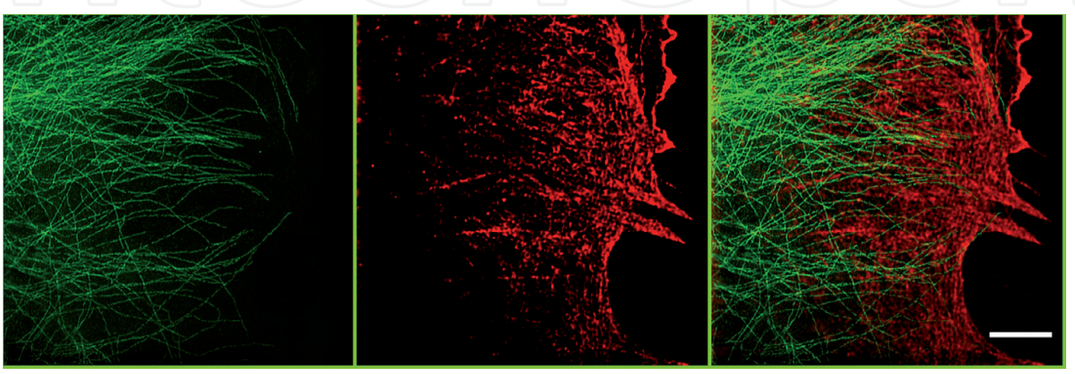

Figure 3.
Microtubules and γ-actin in human venous endothelial cells. Super resolution SIM (structured illumination microscopy) image of cells stained with antibodies against β-tubulin (green) and γ-actin (red). Scale bar, 5 μm. (from: [58] With modification).

3.4 Real-time study of endothelial cells microtubule organization and dynamics

Here, we have already mentioned several times that microtubules are highly dynamic cell component, and the results of our previous experiments demonstrated the critical involvement of the microtubule disassembly in induced endothelial barrier dysfunction [13, 29, 60]. Our previous data allowed us to assume that microtubule dynamics is an early event in the circuit of the reactions leading to the changes in pulmonary endothelial cells barrier permeability [29]. Taking into account the active dynamic properties of microtubules, as well as the ability of microtubule plus-ends tips to act as targeting factors, we proposed to use the microtubule system as a target for the action of substances that cause endothelial barrier dysfunction and on this basis to create a model for studying factors potentially capable to disrupt endothelial layer permeability. For the examination of microtubule behavior in the cytoplasm of endothelial cells, we created and described living cellular model established in physiologically relevant human pulmonary artery endothelial cells (protocol is given below). This model is suitable for a real-time study of microtubule system organization and reorganization in details as well as individual microtubules dynamics (including growth rate of microtubule plus-ends) in the quiescent human endothelial cell monolayer [10] in the normal condition of cultivation as well as under the experimental treatment (in the cells treated with the agents compromising/enhancing endothelial barrier) (**Figure 4**) [9]. Using this model, we were able to obtain several key parameters of microtubule organization in the endothelium such as a ratio between stable and dynamic microtubule subpopulations, direct measurement of microtubule growth rates, and their difference in single endothelial cells and the cells grown as a monolayer [10]. In our opinion, this cellular model would also allow us to study the involvement of microtubules in the barrier-protective/compromising mechanisms activated in pulmonary endothelium by various pharmacological agents of interest.

In this study, we aimed to understand how microtubules contribute to the dynamic reorganization of the endothelial cytoskeleton, we established an endothelial cells model expressing chimeric protein EB3-GFP—microtubule plus ends protein EB3 (a protein that is located on microtubule plus-ends) fused to the fluorescent protein GFP. Using this EB3-GFP microtubule plus-ends marker, we were able to measure microtubule growth rate in different parts of endothelial cell—at the centrosome region and near the cell periphery of a single human endothelial cells or in the endothelial monolayer where cells are adjacent to each other and form cell-cell contacts. This model allows us to show microtubule dynamics heterogeneity of within endothelial cells and to demonstrate that (a) the majority of the endothelial cells microtubules are dynamic and (b) the plus-ends growth rate is highest in the area of the centrosome location, in the internal region cytoplasm. Interestingly, but growth rate of microtubule plus-ends decreases from the cell center toward the periphery. The quantitative data we obtained in the experiments with the single cells and the cells growing in monolayer allow us to suggest the existing mechanism(s) of local regulation of microtubule plus-ends growth in endotheliocytes. It was shown that in the internal cytoplasm of edotheliocytes in the monolayer, microtubule growth rate is lower than that of single endothelial cells suggesting the regulatory effect of cell-cell contacts.

Unexpectedly, the dynamics of microtubules growing from the centrosome in singly located on the substrat surface with no neighbors turned out to be especially original—centrosomal microtubule growth rate distribution in single endothelial cells indicated the presence of two subpopulations of microtubules. One of the populations, the most numerous, included microtubules with “normal” (similar to those in monolayer endothelial cells) and the second population included “fast”

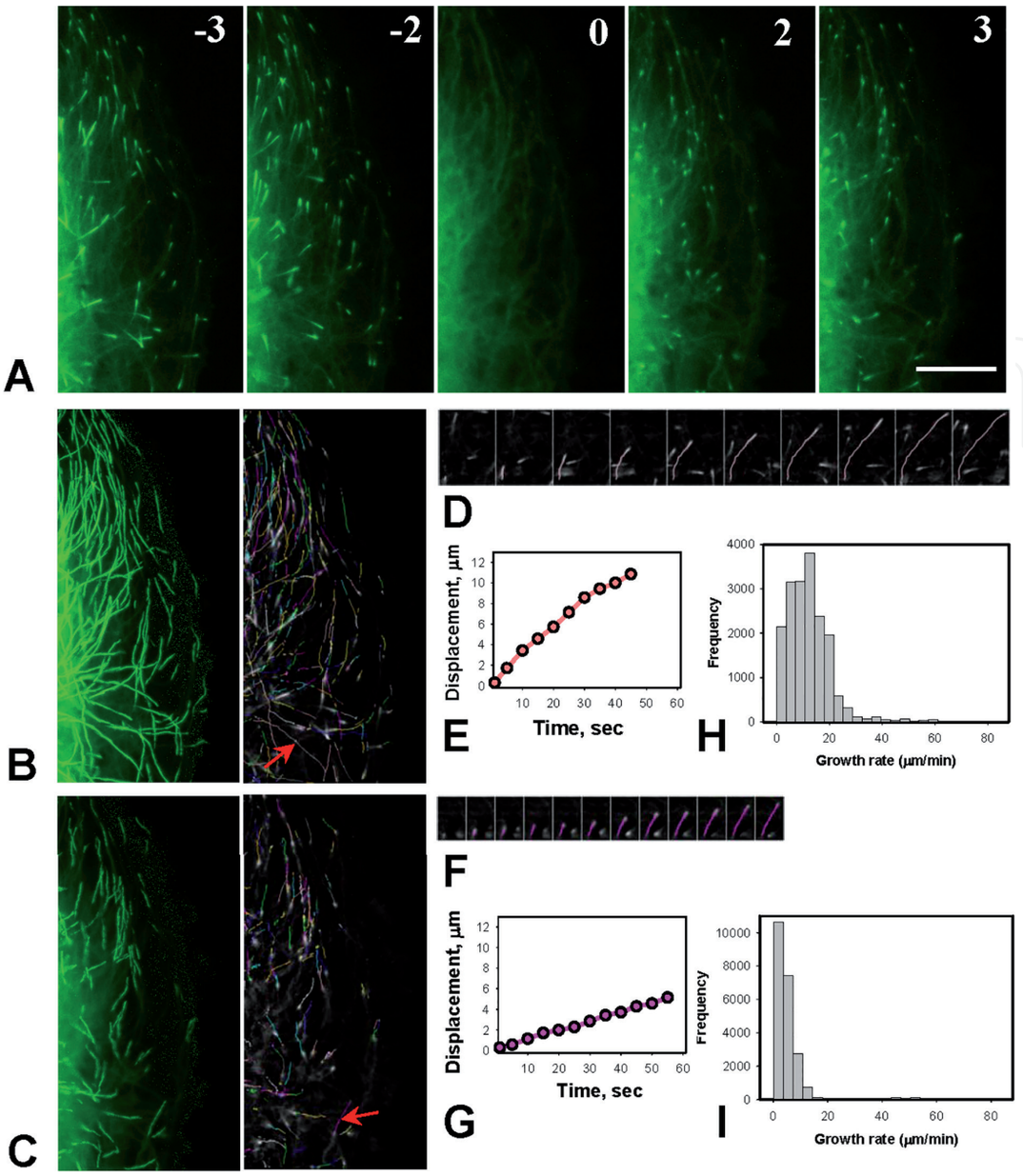

Figure 4.
Real-time study of microtubule dynamics in EB3-GFP-transfected endothelial cells. (A-C). EB3-GFP was used as a marker of growing microtubule plus ends. EB3-GFP movement was examined to analyze the changes of microtubule dynamics during barrier dysfunction EC expressing EB3-GFP were selected for the analysis by time-lapse microscopy (images were acquired every 1 s). (a) EB3-GFP tracks 3 and 2 min before and after nocodazole treatment. EB3-GFP is presented at microtubule plus-ends during growth phases but disappears after transition from growth to pause or shortening phase at the moment of nocodazole application (0 min). Time (min) marked in right top corner. Scale bar, 10 mm. (B, C) EB3 tracks projection obtained by EB3-GFP patches displacement on time-lapse series during 60 seconds (left, tracks projection; right, EB3 tracks are colored individually); between 2 and 3 min before (B) and after (C) nocodazole application. (D, F) Individual microtubules plus end displacement before (D) and after (F) nocodazole treatment (5 s interval between frames). (E) Microtubule plus-ends displacement (red arrow) quantification data are shown in D. (G) Microtubule plus-ends displacement (red arrow) quantification data are shown in F. (H, I) Histograms of microtubule growth rate distribution were obtained by tracking EB3-GFP comets at microtubule plus-ends in HPAEC near the cell margin (H) before nocodazole treatment (mean growth rate, 12.27 ± 0.62 µm/min) and (I) after nocodazole treatment (mean growth rate, 5.76 ± 0.05 µm/min). Thus, the obtained quantitative data allow us to conclude that plus-ends growth rate decreased. (from: [9] With modification).

microtubules, with significantly higher growth rates (threefold as much). Real-time study of endothelial cell microtubule organization and dynamics allowed indicating functional interactions between cell-cell contacts and microtubules. Later in the experiments with nocodazole (anti-microtubule agent, shifting the equilibrium of tubulin polymerization reaction and leading to depolymerization of cytoplasmic

microtubules), it was possible to show that direct microtubules depolymerization by nocodazole initiates the cascade of barrier dysfunction reactions. It turned out that the short-term loss of endothelial barrier function occurs even at the minimal destruction of peripheral microtubules at the time when actin filament system remains intact. Specifically, here, we demonstrate that under these experimental conditions, microtubule dynamics of endothelial cells is very sensitive to external treatment: microtubule plus-ends growth rate has changed and significantly decreased near the cell periphery (**Figure 4**) [9].

3.4.1 Protocol for expression construct and transfection of plasmid

To analyze microtubule dynamics and to calculate the microtubule growth rates in living HPAEC cells, we used expression vectors encoding EB3-GFP (kind gift of Dr. I. Kaverina with permission from Dr. A. Akhmanova), which serves as a marker of growing microtubule plus-ends [61]. For transfection of plasmids into human artery endothelial cells, Effectene transfection reagent (Qiagen Inc., USA) was used according to the manufacturer's protocol. Transfected cells were selected for imaging by GFP fluorescence.

3.4.2 Video microscopy of EB3-GFP-transfected cells

To acquire imaging, human artery endothelial cells were cultured on glass-bottomed dishes with No.1S coverslips (Iwaki, Japan). Images were collected with a PC-based DeltaVision optical sectioning system using one of the following objectives (Olympus): PlanApo 100x/1.40 NA oil or PlanApo 60x/1.40 NA oil ph 3. Images were acquired with a cooled CCD camera (Hamamatsu Photonics) with an appropriate ND filter, binning of pixels, exposure time, and time intervals. Fluorescence signals were visualized using the Endow GFP bandpass emission filter set (41,017, Chroma) for GFP imaging. Quantitative analysis of the microtubule dynamics was carried out on time-lapse movies of cells expressing EB3-GFP. Microtubule growth rates were obtained by tracking EB3-GFP comets at microtubule plus-ends (the shooting frequency was 1 s/frame) using ImageJ software linked to an Excel spreadsheet. Statistical analysis was performed using Sigma Plot 12.5 (SPSS Science, USA).

Modern methods of super-resolution microscopy (stochastic optical reconstruction microscopy [STORM], photo-activated localization microscopy [PALM], and fluorescence photo-activation localization microscopy [FPALM]) especially in combination with another techniques (in particular, Proximity Ligation Assay, etc.) will allow the investigation of living cells structure, so we hope see new interesting results on the cytophysiology of endotheliocytes.

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