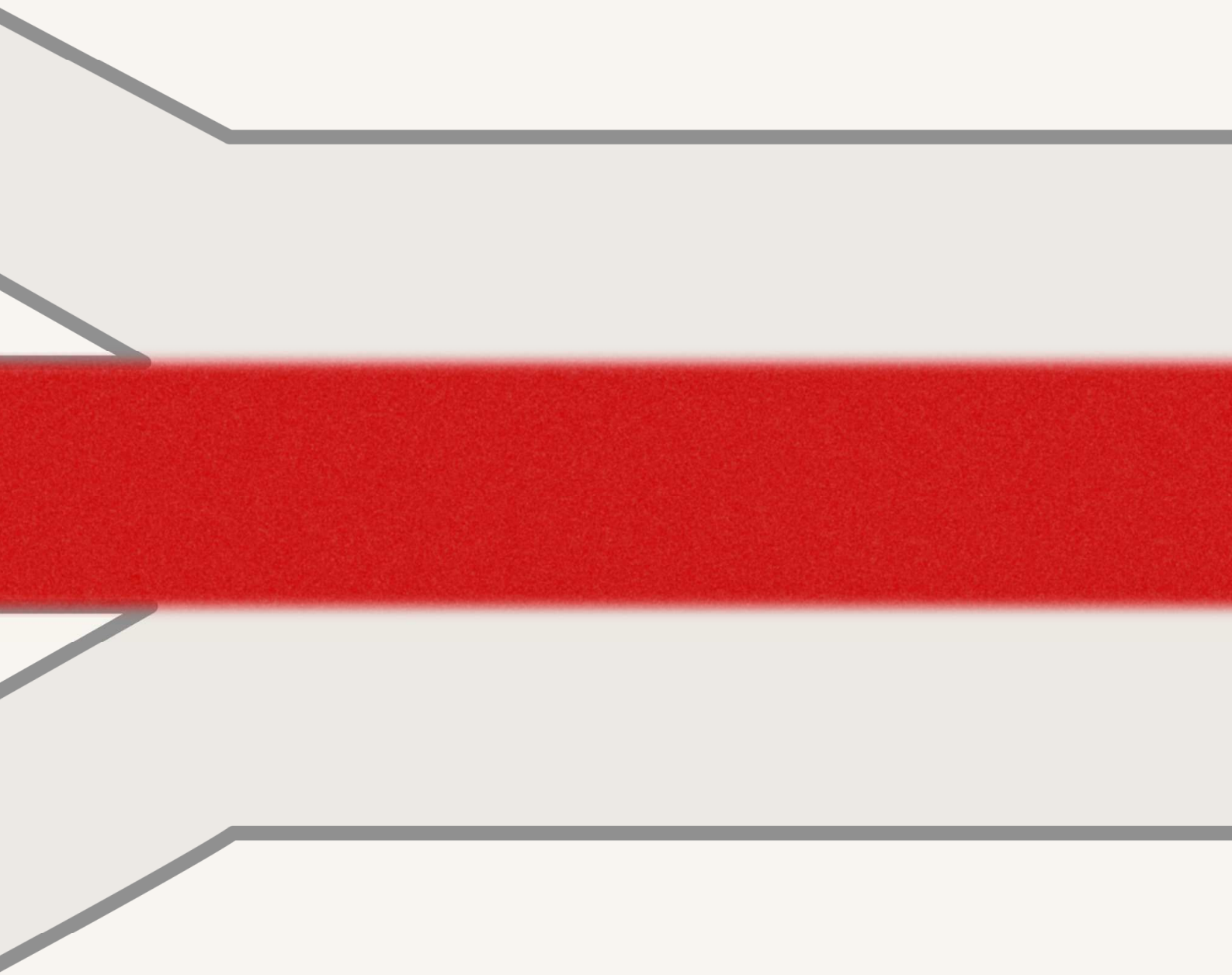
Microfluidic Technology in Vascular Research:

The endothelial response to shear stress



Andries van der Meer

Microfluidic Technology in Vascular Research: The Endothelial Response to Shear Stress

Andries van der Meer

PhD Thesis, with references, with summary in English and Dutch
University of Twente, Enschede, The Netherlands
December 2009

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MICROFLUIDIC TECHNOLOGY IN VASCULAR RESEARCH: THE ENDOTHELIAL RESPONSE TO SHEAR STRESS

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prof. dr. István Vermes
prof. dr. Jan Feijen

en de assistent-promotor:
dr. André Poot

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Samenstelling van de commisie:

Voorzitter	prof. dr. G. van der Steenhoven
Promotor	prof. dr. I. Vermes
Promotor	prof. dr. J. Feijen
Assistent promotor	dr. A.A. Poot
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Preface

Author: A.D. van der Meer

This thesis is the result of a bit more than four years of work, which I performed as a PhD-student in the group of Polymer Chemistry and Biomaterials of the University of Twente in Enschede, The Netherlands. When I decided to accept this position on a late summer's day in 2005, I knew it was a bit of a gamble. I was unfamiliar with the city, the university, and the group with its focus on materials science. Still, it felt right to take this leap into the unknown. Up until that moment, I had never strayed too far from the comfortable security of my childhood friends and family in the vicinity of Groningen, in the north of the Netherlands. The time was ripe for a new challenge. So, after some of prof. István Vermes' typical one-line, staccato-style e-mails and a one-hour talk with my supervisors-to-be, prof. Jan Feijen, dr. André Poot and prof. István Vermes, in an office filled with cigar smoke, the deal was settled. A few weeks later, I moved to Enschede. The following four years would turn out to be a most stimulating experience – both academically and personally.

My project was part of a brand new, in-house, multidisciplinary research orientation that focused on the effect of mechanical stress on living cells. The advantage of a new project is that it allows one to be very actively involved in determining the scope of research. Distilling my own specific research questions from a complex mix of scientific literature, available equipment, in-house expertise, and existing or potential collaborations was a most exciting and educational experience. I have many people to thank for giving me constant feedback during this process: not only my aforementioned trinity of direct supervisors, but also the people that attended the periodical project meetings, like the project leader, Michèl Duits, the external advisor from the hospital, Louis van der Maas, the post-docs (now both assistant professors), Séverine le Gac and Siva Vanapalli and my fellow PhD-student in the project, Jane Li.

During the early years of my project, I travelled a lot from the university campus to the city hospital to perform my experiments. Later on, the group moved to a new building on campus in which all the facilities I needed were clustered. Still, no matter where I worked, I was never without help. Karin Hendriks and Zlata Rekenji were always available when I needed help with administrative issues and purchasing. Moreover, I owe a great deal to the people that helped me find my way in their laboratories, like Remy Wiertz, Paul ter Braak, Yvonne Kraan, Kirsten van Leijenhorst and Judith Olde Wolbers, or that collaborated with me intensively in my project, like Séverine le Gac, Floor Wolbers, Daniël Wijnperle and Jane Li. Moreover, I was lucky

enough to supervise some very enthusiastic bachelor students in their scientific projects: Tyron Jermin, Fikri Abali, Sieger Henke and Marco Timmer. All of them performed very useful exploratory work within my project. Still, I owe the most to the two people that collaborated with me on a daily basis and that contributed a great deal to the practical work that is described in this thesis, the technicians Marloes Kamphuis and Kim Vermeul.

It was predominantly in the last year of my project that I summarized my findings in the scientific papers that form the body of this thesis. Writing these papers has been a fun and fulfilling experience for me; even though, judging by the general opinion at the group's coffee table, this makes me somewhat of a rarity. During the process of writing, I received a lot of feedback on both content and style from my supervisors, André, István and Jan, as well as from the project leader, Michèl. I am very grateful for their help; it has greatly improved the quality of my work.

The completion of this thesis marks the end of my training as a scientist. I would have never completed this training so successfully and happily if it wasn't for the great personal atmosphere at the university. I would like to thank all the colleagues and friends that have made my stay in Enschede so pleasant, both at and outside of work. Objectively – as a biologist from another town and another university – I might have been a bit of an outsider, but I never was and I never felt that way.

Andries van der Meer
December 2009, Utrecht, The Netherlands

Chapter 1: Introduction

Authors: A.D. van der Meer, A.A. Poot, J. Feijen, I. Vermes

Background

Microfluidic technology

Microfluidic technology deals with the manipulation of nanoliters of fluid in channels with dimensions ranging from tens to hundreds of micrometer.[206] However, microfluidics is about more than the simple pumping of fluid through a micrometer-sized capillary. The technology is a lot more versatile, because complete control over the fluid streams is possible by mixing, switching and pumping. This means that the technology can be used to develop complex, electronic-microchip-like systems, with numerous channels that can be controlled individually or as a group.[180]

Research activity in the field of microfluidics has increased dramatically over the last few decades. This has led to a number of interesting applications, like increased throughput in screening for protein crystallization conditions,[216] new methods for analyzing biomolecules,[152] and faster screening in drug development.[40]

Because almost all microfluidic devices are constructed of materials that are non-toxic to cells, like glass and the transparent, gas-permeable elastomer polydimethylsiloxane (PDMS), the devices can also be used in the field of cell biology. When applying microfluidic technology in this field, the immediate advantages are obvious: when using small channels, smaller amounts of cells and reagents are needed than when using conventional set-ups, and throughput can be increased by parallelization.[204] Moreover, in the future, it may be possible to produce a microfluidic system that combines cell culture, treatment, lysis and multiple forms of analysis in one device: the micro-total-analysis-system (μTAS) or 'lab-on-a-chip'. A lot of proof-of-concept work for parts of such μTAS systems has already been reported in literature.[44]

The application of microfluidic technology in the field of cell biology is promising. However, the technology is not yet used as widely as may have been anticipated by engineers in the field of microfluidics. There are a number of possible explanations for this, which were also recently highlighted by Paguirigan and Beebe in a scientific essay.[134] First of all, changes in the cell culture micro-environment can have a big impact on cell physiology. Substrate stiffness and surface energy, surface coating and non-specific adsorption of proteins, surface-to-volume ratios, hydrodynamic forces, they all have their impact on cell behavior. With the widely used tissue-culture polystyrene flasks and wells-plates, these properties are either well-known or taken to be constant and independent from cultureware producer or cultureware format. Therefore, experiments that are performed with these conventional products do not

raise questions about the validity of the cell culture system. In the case of microfluidic devices, these aspects of surface chemistry and micro-environmental physics are not simply taken for granted: biological validation of the devices is needed. Second of all, microfluidic devices can become quite complex and difficult to work with. Most devices need large amounts of well-calibrated pumps, syringes, tubing and connections to carry out their functions. Microfluidic devices may be small and elegant, but bulky and relatively complex set-ups are currently needed to control them. The third issue with microfluidic devices is that most of them are incompatible with standard biochemical technology for read-out. Biologists have a wide range of biochemical techniques at their disposal, like electrophoresis and blotting of proteins and nucleic acids, colorimetric and fluorescent assays for 96- and 384-wells plates and magnetic or fluorescence-activated cell sorting. Not all of these read-out techniques are directly compatible with microfluidic devices, limiting the information that can be obtained by performing a microfluidic experiment.

In summary, microfluidic technology holds great promise for improving and facilitating cell biological research. However, application of the technology to the field of cell biology is still hampered by practical problems.

Fluid shear stress and the endothelial response

One of the areas of cell biological research where microfluidic technology may be successfully applied is in studying the response of vascular endothelial cells to fluid shear stress. Endothelial cells form the inner lining of all blood vessels and have important functions in regulating blood clotting, transport of nutrients and waste products, vasodilation and vasoconstriction, inflammation and angiogenesis.[24] Interestingly, the physiology of endothelial cells is strongly affected by the shear stress exerted on them by blood flowing over their surface.[107] The effects of shear stress on endothelial physiology are under intensive research, because of the relevance to vascular diseases like atherosclerosis. Atherosclerosis – chronic inflammation and thickening of the blood vessel wall – is localized predominantly in regions of the vascular tree with disturbed, irregular blood flow patterns.[60] Endothelial dysfunction is an important step in the initiation and progression of this disease.[115] So understanding how mechanical stress affects endothelial physiology will lead to insight in the pathophysiology of atherosclerosis and may provide inspiration for new treatment strategies.

Aim and outline

The aim of this thesis is to generate more insight into the response of vascular endothelial cells to fluid shear stress by using microfluidic technology. This is achieved by culturing human endothelial cells in microfluidic channels and subse-

quently pumping medium through the channels at a rate that imposes a physiologically relevant fluid shear stress on the cells. Using this approach, a number of aspects of the varied response of endothelial cells to shear stress is addressed in the studies described in this thesis. Moreover, the thesis contains examples of successful application of microfluidic technology in the field of cell biology, addressing some of the aforementioned issues with respect to combining microfluidics and cell biology.

Chapter 2 of this thesis gives an introduction to the field of microfluidic technology, with a strong focus on applications in vascular science and the available literature in this multidisciplinary field of research.[195]

Chapter 3 gives an example of a typical study on signal transduction in endothelial cells without using microfluidic technology.[192] It serves as a point of reference to the reader to understand how conventional in vitro vascular research is performed. In the study, it is shown that the membrane scaffolding protein caveolin-1 is essential for activation of vascular endothelial growth factor receptor-2 and subsequent initiation of signal transduction in response to fluid shear stress.

The study in **Chapter 4** focuses on the cytoskeletal remodeling in endothelial cells in response to fluid shear stress. By using a microfluidic assay, the signal transduction pathways involved in this functional response can be investigated in a quick and easy fashion. By applying the assay, it is shown that vascular endothelial growth factor receptor-2 and Rho-associated kinase are needed for the remodeling, while the protein kinase B/Akt pathway is not involved. Moreover, this study uses a simple approach to validate the microfluidic assay by reproducing results from studies in the same field.

Chapter 5 gives a typical illustration of one of the advantages of microfluidic technology, namely its suitability for a combination with high magnification, live cell, fluorescence microscopy. In this study, a set-up for intracellular, sub-micrometer-sized particle tracking is adapted to study the immediate micromechanical response of endothelial cells that are subjected to fluid shear stress. It is shown that fluid shear stress induces an immediate, but transient micromechanical stiffening in endothelial cells and that this effect is dependent on activation of vascular endothelial growth factor receptor-2.

In **Chapter 6**, endothelial cell migration is studied by setting up a microfluidic version of the wound healing assay and comparing it to the conventional assay.[191] The main benefits of the microfluidic assay are that fluid shear stress and growth factor gradients can be applied during wound healing. By taking advantage of these features, it is shown that the wound healing rate is higher when shear stress or a gradient of vascular endothelial growth factor is applied to the cells. This chapter illustrates a few ways to induce increased acceptance of microfluidic technology. First of all, characterization of the microfluidic system and comparison of the results with

results from conventional methods increases the trustworthiness of the microfluidic assay and its results. Moreover, stable growth factor gradients are impossible to establish in the conventional assay, forcing other biologists to also adopt microfluidic technology in order to replicate and build on the reported results.

In the study described in **Chapter 7**, microfluidic technology is combined with conventional flow cytometry. Using this merged protocol, it is shown that the uptake of low density lipoprotein by endothelial cells is significantly lower when the cells are pretreated with fluid shear stress. This study bridges the gap between microfluidic technology and a conventional read-out technique. By demonstrating the compatibility of the two fields of technology, the threshold for acceptance of microfluidic technology can be lowered.

In **Chapter 8**, the most important conclusions of the thesis are highlighted and an outlook on future work is given.

In the **Appendix**, the first results of developing a microfluidic assay to follow the uptake of siRNA-containing nanoparticles are presented.[193] Instead of following this process in static cell cultures, it is more realistic to deliver the particles under flow. Performing the assay under dynamic conditions leads to decreased particle uptake in comparison to static conditions.

Chapter 2: Microfluidic technology in vascular research

Published: A.D. van der Meer, A.A. Poot, M.H.G. Duits, J. Feijen, I. Vermes, Journal of Biomedicine and Biotechnology 2009:823148, 2009

Vascular cell biology is an area of research with great biomedical relevance. Vascular dysfunction is involved in major diseases such as atherosclerosis, diabetes and cancer. However, when studying vascular cell biology in the laboratory, it is difficult to mimic the dynamic, three-dimensional microenvironment that is found in vivo. Microfluidic technology offers unique possibilities to overcome this difficulty. In this review, an overview of the recent applications of microfluidic technology in the field of vascular biological research will be given. Examples of how microfluidics can be used to generate shear stresses, growth factor gradients, co-cultures and migration assays will be provided. The use of microfluidic devices in studying three-dimensional models of vascular tissue will be discussed. It is concluded that microfluidic technology offers great possibilities to systematically study vascular cell biology with set-ups that more closely mimic the in vivo situation than those that are generated with conventional methods.

Introduction

Vascular science is an active area of research. Scientists world-wide are trying to unravel the mechanisms that determine vascular function and dysfunction. Important objects of study in this field of research include the maintenance of vascular tone,[46] regulation of inflammation,[101] sprouting of new blood vessels,[49] regulation of cell survival[43] and the differentiation of stem cells into vascular tissue.[88] Vascular science is a field with a strong translational focus, combining results from fundamental molecular and cell biology with in vitro models of blood vessels and in vivo tests to develop insight in vascular physiology and treatment of disease. Vascular dysfunction is an important factor in major diseases like atherosclerosis,[32] cancer[66] and diabetes.[25] The basis for understanding the functioning of blood vessels lies in understanding its building blocks, the vascular cells. Therefore, a lot of research is focused on how endothelial cells or smooth muscle cells react to relevant biological, chemical, or physical cues in vitro. Usually, this work is carried out by using conventional methods, culturing cells of animal or human origin in wells-plates, subjecting them to the aforementioned stimuli and analyzing the outcome by biological or biochemical techniques. However, in vivo, dynamic conditions are present: vascular endothelial cells are constantly subjected to shear stress caused by the flowing blood,[107] while

smooth muscle cells are stretched because of distension of the blood vessel during the cardiac cycle.[59] Moreover, vascular cells are embedded in a three-dimensional environment consisting of an elastic extracellular matrix,[33] other cells[36] and flowing blood, with its platelets,[150] red blood cells,[138] and leukocytes (figure 1).[143] Both the three-dimensional environment and the dynamic mechanical changes with each cardiac cycle are very important factors in vascular cell functioning. It is advantageous to design laboratory set-ups that allow researchers to include these factors and control the relevant parameters. The main challenge when building such set-ups is that they should still be easy to assemble, handle and combine with conventional analysis techniques.

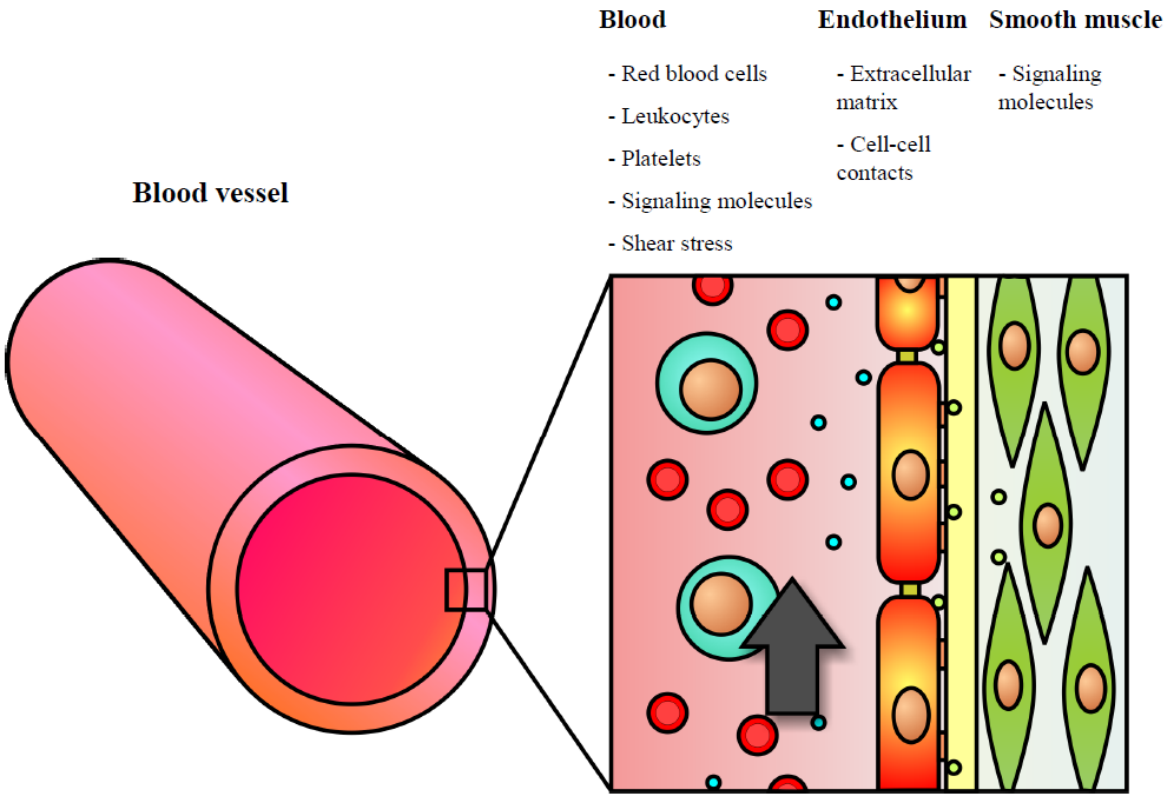

Figure 1: Schematic overview of a blood vessel and the endothelial cell microenvironment. The inner wall of a blood vessel (left) consists of a layer of endothelial cells that are embedded in a three-dimensional microenvironment (right). This environment consists of cell biological, biochemical and physical stimuli, such as red and white blood cells, signaling molecules and shear stress, respectively. Mimicking this complex microenvironment in vitro is a major challenge in vascular research.

In the recent years, the field of microfluidic technology has gained much scientific interest among biologists, biochemists and biophysicists (figure 2). We feel that microfluidic technology holds great promise to overcome the challenge of perform-

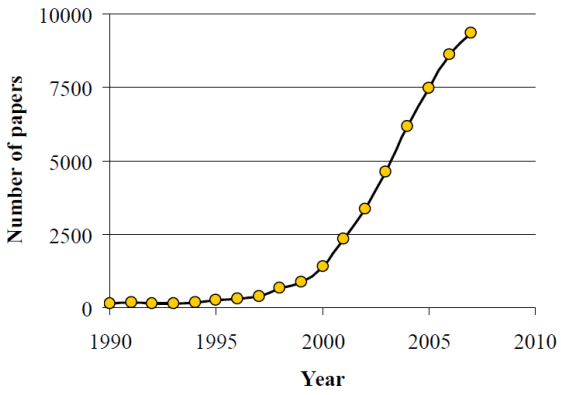

Figure 2: Graph of the amount of papers containing the keyword 'microfluidic' that were published per year as determined with Google Scholar. A more than 30-fold increase in the yearly number of scientific publications with this particular keyword can be observed in the decade between 1995 and 2005.

ing in vitro experiments with more physiologically realistic set-ups that are still simple enough to be used in everyday laboratory practice. Moreover, microfluidic technology allows for increasing scale and parallelization of current research, leading to more comprehensive insights into cell and tissue physiology. The advantages of microfluidic technology for cell culture in general have been reviewed elsewhere.[44, 123] In this review, we will focus specifically on the application of microfluidic devices in vascular cell biology research.

Microfluidic technology

Fabrication

Microfluidic technology deals with the design, fabrication and application of devices for manipulation of fluids on the micrometer-scale. Typically, the sizes of features in these devices range from several micrometers to a few hundred micrometers. The amounts of fluid that are manipulated inside these devices are typically in the picoliter to nanoliter range. Microfluidic devices can be fabricated using metal, glass or polymer materials. Most devices that are used in combination with cell biological research are made of glass or the silicone rubber polydimethylsiloxane (PDMS), because these materials are cheap, biocompatible and transparent.

Because all microfluidic studies that are discussed in this review use microfluidic devices of PDMS (sometimes combined with glass components), the process of producing these devices will be described shortly (see also figure 3). PDMS devices are produced by soft lithography replica molding,[42] which means that the devices are elastic replicas of a stiff, re-usable mold. The process starts by producing the stiff mold with the desired structures. The mold is usually made of silicon with micrometer-size structures produced either by plasma-etching of the silicon plate or by building on top of the plate with the epoxy-based, photo-crosslinkable polymer SU-8. A mixture of PDMS oligomers is poured on top of this mold, allowed to solidify by crosslinking and then peeled off from the mold. In order to create sealed channels,

the surface of the PDMS replica is activated with oxygen plasma and bound to a PDMS or glass surface. Holes can be punctured to reach the closed channel structure and tubing can be connected to manipulate fluid inside the channels. The silicon master-molds need to be produced in a clean room, but replica molding can be performed under standard laboratory conditions. Once the master-mold has been created, producing new microfluidic devices by this method takes only a few hours. Because the materials are cheap, microfluidic devices can be discarded after every experiment.

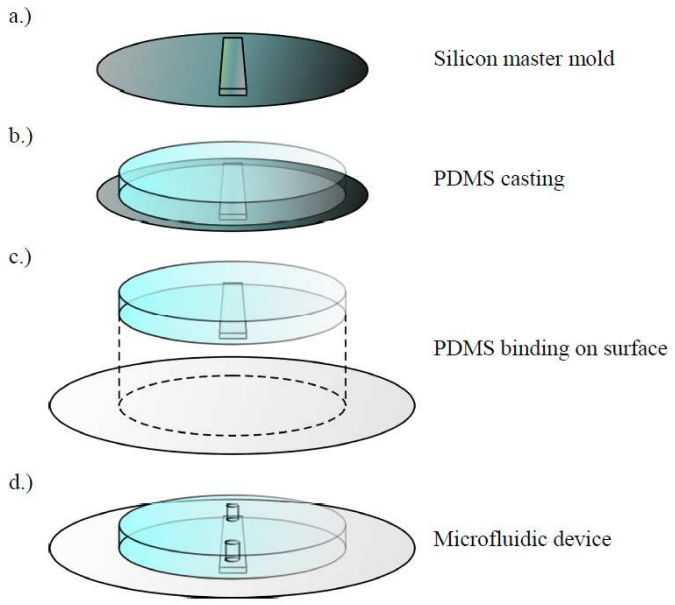

Figure 3: Schematic overview of the fabrication of PDMS chips. a.) The process starts by fabricating a silicon master mold – with typical dimensions of 10 centimeter in diameter and 0.5 millimeter thickness – with microfluidic structures on top. b.) A viscous mixture of PDMS oligomers and crosslinker is poured on top of the mold and allowed to form a flexible, crosslinked network. c.) The slab of PDMS is peeled off of the mold and bound to a glass or PDMS surface to produce closed microfluidic channels. d.) The microfluidic device is ready to be used. Prior to binding to the surface, holes can be punched in the slab of PDMS to reach the resulting microfluidic channel.

Cells and microfluidic technology

Generally speaking, PDMS microfluidic devices offer a number of distinct advantages over conventional techniques for cell culturing, manipulation and analysis. The main feature of microfluidic devices that makes them suitable for use in cell biology is that they are smaller than conventional setups (for an impression of the size of a microfluidic channel, see figure 4a). Because of this small size, only limited amounts of cells, media and reagents are needed. This leads to a number of significant benefits. First of all, if experiments are to be conducted with rare primary cell material or expensive drugs, it is quite advantageous to use only small quantities of these valuable materials. Secondly, if cultures are to be maintained under conditions of constant fluid flow, small sizes are a considerable advantage. In conventional bioreactors, cell culture medium is usually collected and re-used after it has passed through the cell culture chamber. The medium is then completely replaced every few days. In microfluidic devices, a constant flux of fresh medium can be used, because the volumes

involved are orders of magnitude smaller. The third benefit is that the small, planar and transparent microfluidic set-ups are easily combined with bright field and fluorescence microscopy or spectroscopy, because they fit easily on stages of conventional microscopes. This facilitates monitoring cell behavior for long periods and with high magnification during the experiment.

It is important to realize that cells that are cultured inside microfluidic devices need to be subjected to a constant flux of fresh medium. When the small volumes in the cell-containing devices would be left under static conditions, nutrients would be depleted quickly, whereas waste products would increase to undesirable concentrations. The fact that constant refreshment of medium is needed may seem cumbersome at first glance. However, under physiological conditions, all cell types need a flux of nutrients and waste products. The flow conditions in microfluidic devices mimic this process more closely than in vitro culturing in wells-plates.[86]

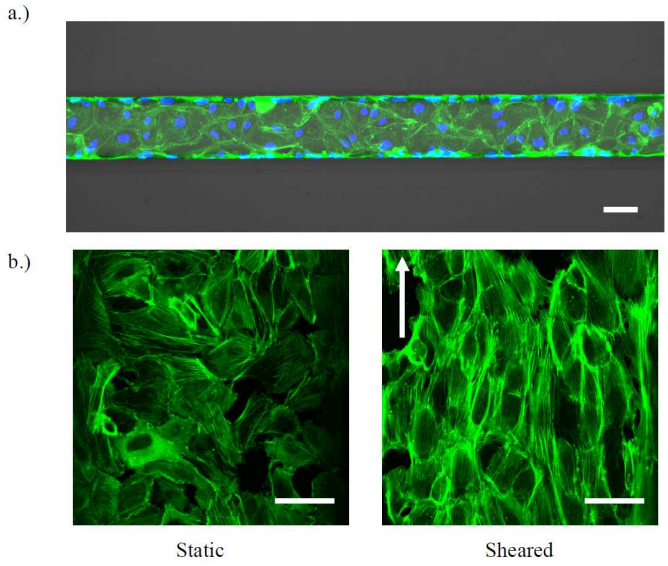

Figure 4: Endothelial cells in a microfluidic channel. a.) Human umbilical vein endothelial cells were cultured in a microfluidic channel. When reaching confluence, the cells were fixated with paraformaldehyde, and stained for actin filaments (green) and nuclei (blue). Scale bar is 50 μm. b.) Endothelial cells that are subjected to physiological levels of shear stress inside microfluidic channels reorient their actin cytoskeleton to align with the direction of fluid flow (right). Scale bars are 50 μm.

Because of the small dimensions of microfluidic channels, fluid flow is fully laminar, meaning that the flow patterns are completely predictable and turbulent mixing does not occur. In some applications, such as microreactors, which require mixing of different reagents, this laminar flow pattern is an obstacle that has to be overcome. However, the laminar nature of the fluid flow can also be used to perform unique experiments that are difficult or nearly impossible to perform with conventional methods. Most of these experiments rely on parallel fluid flows: if two streams of fluid enter the chip in a parallel fashion, the two streams will remain separated and mixing of them will only occur by diffusion. Thus, the degree of mixing can be tuned by changing the flow rate: the higher the flow rate, the shorter the residence time inside the device, the less the streams mix. Therefore, as long as flow rates are sufficiently high, cells on one side of the device can be treated with

one substance, whereas cells on the other side are treated with another substance. As a matter of fact, even two sides of a single cell can be treated in this way.[176]

Another important main feature of microfluidic technology is that it is suitable for high-throughput, comprehensive studies of cell biology. This means that the effect of multiple factors and parameters on cell functioning can be screened in one assay. Increasing throughput is an active area of research in the field of microfluidics. Efforts are made to merge microfluidic technology with microarray and microtitre plate technology.[165, 178, 182] Also, researchers are dedicated to integrate multiple steps, such as cell culturing, lysis and analysis in one device. Numerous examples of this parallel and serial microfluidic biochemical analysis, also known as lab-on-a-chip, have already been reported and are starting to be implemented in cell-containing microfluidic devices.[44]

Microfluidic technology and vascular cells

The endothelial mechanoresponse

Vascular endothelial cells are highly responsive to shear stress that is caused by the flow of fluid over their surface. This shear stress is the result of the presence of a fluid velocity gradient in the cross section of a tube. The velocity of the fluid next to the walls is zero, whereas the velocity is maximal in the center of the channel. The steeper this gradient, the higher the shear forces that act on the vessel wall. The biological response to this mechanical stimulus – the endothelial mechanoresponse – has been found to be a key process in preventing vascular disease.[28] The mechanoresponse is usually studied in vitro by subjecting endothelial cells to shear stress in parallel plate flow chambers. Microfluidic devices can be considered as miniaturized versions of these set-ups. Because shear stress is proportional to flow rate and inversely proportional to channel dimensions, only low flow rates are needed in microfluidic channels to mimic the high shear stresses found in the human body. Song, et al.[168] took advantage of this fact by designing a microfluidic device that can subject endothelial cells to physiological levels of shear stress in multiple parallel channels. They showed that a flow rate of less than 200 µl per hour is already enough to make the sheared endothelial cells elongate and orient in the direction of the flow, which is a prominent feature of the endothelial mechanoresponse that is also found in vivo. This reorientation is also reflected in the actin cytoskeleton of the cells. In our laboratory, we subjected cells to a shear stress of 1 Pa for 12 hours and then stained the actin filaments with phalloidin-FITC. Most filaments were aligned and oriented in the flow direction (figure 4b). Recently, Tkachenko, et al.[181] also reported the design of a microfluidic device that allows for real-time tracking of endothelial cells that are subjected to shear stress. They could generate shear stresses ranging from 0.01 to 0.9

Pa in parallel channels, using flow rates in the range of several milliliters per hour. In contrast, flow rates are in the order of hundreds of milliliters per hour for the conventional, larger, parallel plate flow chambers. Because of the small volumes of reagents that are needed, and the potential parallelized design of microfluidic devices, they are an ideal platform for screening of compounds that may have an impact on the mechanoresponse. We have recently developed such an assay, in which the morphological rearrangements of endothelial cells are used to quantify the mechanoresponse. Using this assay, the impact of inhibitory drugs on the mechanoresponse can be detected (see Chapter 4 of this thesis).

Another well-known effect of applying shear stress to endothelial cells is the release of the vasodilatant, nitric oxide.[94] Microfluidic assays have already been reported that can detect the production of nitric oxide in response to chemical stimuli amperometrically[171] or by fluorescence.[130] This provides researchers with an interesting tool to study nitric oxide release in response to both mechanical and chemical stimuli.

When increasing the flow rate and the resultant shear stress, microfluidic devices can also be used to study the adhesion strength of endothelial cells to their underlying substrate. Young et al.[212] performed such an experiment with endothelial cells of different origins and two types of matrix proteins. When the cells were subjected to a shear stress that is about ten times higher than typical physiological values, a certain percentage of cells detached from the surface. In this manner, it was possible to give a semi-quantitative indication of the strength of adhesion of different cells on different substrates. These types of experiments used to be performed with large, parallel plate shear devices that consumed large amounts of media, cells and reagents.[188] Downscaling of these set-ups to micrometer dimensions is a clear advantage.

Migration assays

As discussed earlier, multiple parallel fluid flows can be introduced in one microfluidic channel. Transport of components from one flow to the other only occurs by diffusion (figure 5a). If flow rates are low, there is sufficient time for the parallel streams to exchange components. If one of the streams contains a drug or active compound, stable gradients can be generated by taking advantage of this diffusion. An example of such a gradient that was produced in our laboratory is shown in figure 5b. There are a number of studies that show how this phenomenon can be used when experimenting with vascular cells. Most of these studies focus on migration of vascular cells in response to gradients of physical or biochemical cues. Studying and understanding cell migration is important, because it is a process involved in embryogenesis, wound healing and tumorigenesis.

Barkefors et al.[10] studied migration of endothelial cells in gradients of vascular endothelial growth factor ($VEGF_{165}$). They designed a device with three inlets, generating three parallel fluid streams in the main channel. When $VEGF_{165}$ was added to the middle stream, an increasing gradient from the sides of the channel towards the middle was generated. The steepness of this gradient could be tuned by adjusting the flow rates: the slower the flow rate, the longer the residence time in the channel, the more time there is for $VEGF_{165}$ to diffuse and the more shallow the gradient becomes. When endothelial cells were cultured in this stable gradient of $VEGF_{165}$, they preferentially migrated towards the middle of the channel. Because the researchers had control over the shape of the gradient, they could show that steep gradients induce faster migration. Moreover, it was found that endothelial cells migrated fastest in gradients from 0 to 50 ng/ml, whereas they were not able to sense gradients from 50 to 100 ng/ml due to saturation of the available receptors.

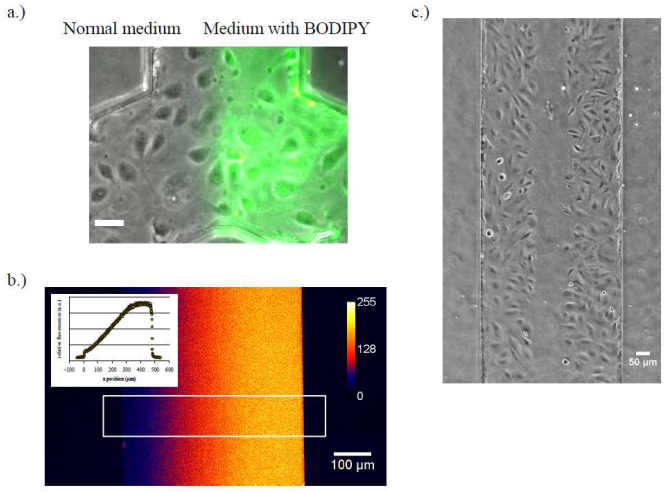

Figure 5: Uses of parallel fluid flows inside microfluidic channels. a.) Parts of the microfluidic channel can be treated differently by pumping two types of media into the two inlets. In this case, a fluorescent label was added to one of the parallel fluid streams. Flow is from bottom to top, scale bar is 50 µm. b.) When flow rates are sufficiently low, media reside in the channel long enough for diffusion to take place. This phenomenon can be used to generate and maintain steady gradients in a channel. In this case, three parallel inlet streams were used, containing 0 µg/ml, 5 µg/ml and 10 µg/ml dextran-rhodamine, respectively. When quantifying the fluorescence over the width of the channel, an almost linear gradient can be observed (white square box in the image, plotted in the inset). c.) By using parallel flows, the middle part of the channel was treated with trypsin. As a result, endothelial cells in the middle of the channel are selectively removed, creating an artificial wound. The closing of this wound can be followed over time to quantify cell migration rates.

Biochemical cues, such as the growth factors used in this study, are not the only relevant stimuli for vascular cell migration. In an elegant study, Zaari et al.[215] showed that smooth muscle cells tend to migrate towards mechanically stiffer underlying substrates. To reach this conclusion, the authors designed a microfluidic device that could generate a gradient of crosslinker, mixed with a solution of acrylamide. A layer of gel with a gradient of stiffness was produced by crosslinking the mixture with UV light into a polyacrylamide network. When these gels were taken out of the microfluidic devices and smooth muscle cells were seeded on them, all cells tended to migrate towards the side of the gel with higher stiffness.

Apart from migration assays that rely on gradients, the parallel laminar fluid streams can also be used to bring the most conventional migration assay to a microfluidic scale. This assay is the scratch assay, or wound healing assay. It works by growing cells in a monolayer, artificially creating a scratch and then following how this scratch is closed by directed migration of the surrounding cells. In a microfluidic device, the artificial scratch can be generated by adding the serine protease trypsin to one of the parallel fluid streams. When one side of the channel has been cleared of cells by trypsinization, the migration of the remaining cells can be followed over time to quantify directed migration. So far, this assay has only been published with data on fibroblasts,[129] but work in our group has shown that it is also possible with endothelial cells (figure 5c; Chapter 6 of this thesis). The advantage of carrying out this assay in a microfluidic device is that it can be more easily combined with stimuli such as shear stress or growth factor gradients.

Cell interactions

The principle of parallel streams is not just useful in studies of cell migration. It can also be used to pattern cells inside a microfluidic device. This is important when interactions between cells are the object of study. Micrometer-scale patterning of cells can be achieved by stamping adhesive proteins on a substrate[209] or by temporarily confining cells in a microfluidic device until they adhere, after which the device is removed from the surface.[23] By using parallel streams, cells can be patterned without the need of removing the microfluidic device afterwards. When adding one cell type to one stream and another type to the parallel stream, cells can be co-cultured in direct contact with each other inside a microfluidic device.[175] For vascular research, this method could be used to pattern endothelial cells and smooth muscle cells in one device. The planar nature of microfluidic devices would provide great opportunities for studying interactions between these cell types. In literature, there are numerous reports of microfluidic set-ups that are used for vascular cell interaction studies. For example, Song, et al.[167] studied the interaction between endothelial cells and circulating tumor cells, a process that is important for cancer metastasis. They developed a device in which a layer of endothelial cells can be stimulated with chemokines from the bottom, while being treated simultaneously with a suspension of breast cancer cells from the top. When the endothelium was stimulated with CXCL12, a chemokine implicated in metastasis, they found that more cancer cells adhered to the layer of endothelial cells than under basal conditions. Another study on metastasis used a microfluidic chip with small, gel-coated gaps, overlaid with a monolayer of endothelial cells to mimic the basement membrane and the endothelium, respectively.[22] Using this microfluidic in vitro model of a blood vessel, tumor cell migration could be quantified and studied in great detail with time-lapse micros-

copy. It is not just interactions between tumor cells and endothelium that are an interesting object of research in vascular science. Studying the interactions between other circulating cells and endothelial cells is also important. For example, the binding of leukocytes to endothelial cells is an essential step in inflammation,[4] while the endothelium-mediated activation of blood platelets is important in clotting and thrombosis.[6] Multiple reports have been published by groups that studied the adhesion of leukocytes[156] or platelets[58, 92, 127] to endothelial cells or endothelial cell-derived adhesion factors in microfluidic devices. These reports show that microfluidic cell interaction studies require less sample and reagents than similar, conventional studies. Moreover, a number of these studies already show increased throughput by using parallel channels in one device.[58, 92, 156]

Three-dimensional culturing

An important factor in vascular cell physiology is its three-dimensional microenvironment. Cells are embedded in an environment that comprises other cells, extracellular matrix proteins, bodily fluids and blood. Three-dimensional cell culturing in the laboratory can be performed by incorporating cells in a hydrogel matrix (e.g. the commercially available, collagen-based Matrigel), or by growing cells on top of this matrix, allowing them to migrate into the gel.[189] Still, the complex real-life, three-dimensional microenvironment is usually reduced to a two-dimensional system when experiments are carried out on cells in vitro. This is more convenient, because with such a system cells can easily be supplied with fresh growth medium, growth factors and other soluble compounds. Moreover, a two-dimensional set-up is more compatible with microscopy and imaging. However, when using microfluidic devices, replenishment of medium, generation of gradients and microscopic imaging is relatively easy to realize in a three-dimensional culturing environment. A good example is the recent publication by Vickerman et al.[200] They describe a microfluidic device with two parallel channels, connected by a gel chamber. The gel chamber is filled with a collagen based hydrogel and endothelial cells are grown in one of the channels. By generating a gradient of soluble growth factors, the endothelial cells grow into the gel and even form open capillaries that span the entire gel chamber from channel to channel. In this particular article, the gel is pipetted into the gel chamber by microinjection before assembling the device. However, using the laminar flow properties discussed earlier in this review, hydrogels can also be formed in situ and even be patterned and confined to certain regions of the microfluidic device.[87] The great potential of these three-dimensional culturing techniques was recently underlined by Barkefors et al.,[11] who cultured ex vivo kidney tissue and followed the formation of blood vessels in response to a $VEGF_{165}$ gradient. Because of the small scale of microfluidic devices, it is possible to advance this proof-of-concept study

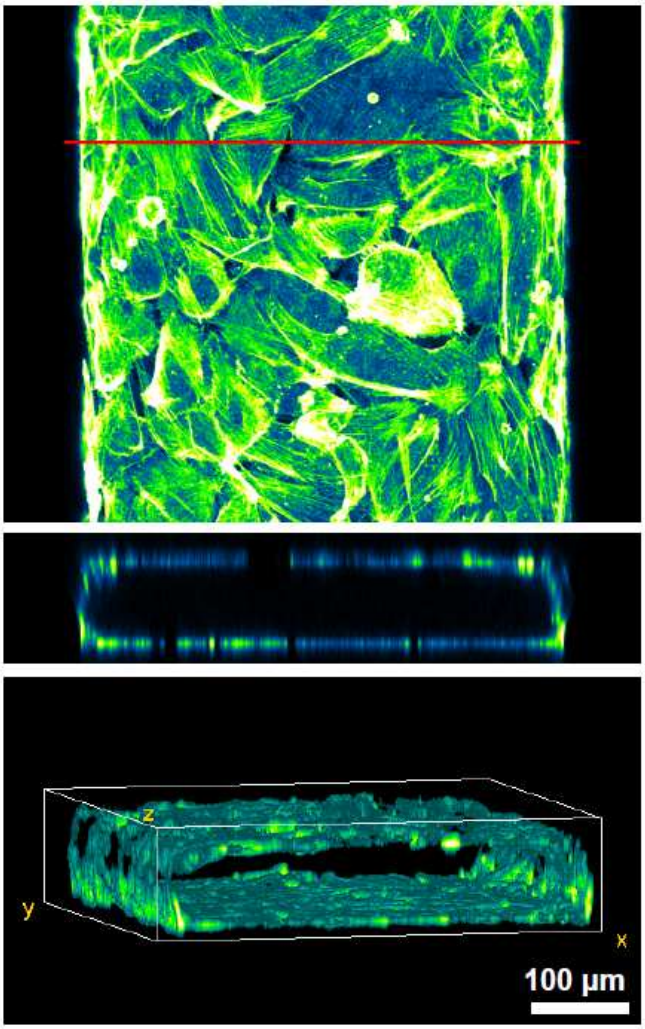

Figure 6: Covering all surfaces of a microfluidic channel yields 'artificial capillaries'. Human endothelial cells were cultured in a PDMS microfluidic channel and allowed to cover all surfaces. After overnight culturing, cells were fixated and actin filaments were imaged with confocal laser scanning microscopy. Top image is a pseudo-colored top view of the microfluidic channel. The red line marks the section that was used to construct a front view of the channel (middle image). The bottom image is an isometric volume view of the same channel.

towards high-throughput assays in order to screen for compounds that affect blood vessel formation in such realistic models. A good example of this high-throughput trend is the recent study by Hsiao et al.,[69] who studied three-dimensional, spheroid co-cultures of prostate cancer cells and endothelial cells in a microfluidic device with 28 side chambers that could all harbor a tumorous spheroid.

Compound screening assays

In biomedical engineering, a lot of research is dedicated to developing particle systems that carry drugs, proteins, DNA for gene therapy or siRNA for gene silencing to their proper site of action. In this field of research, it is important to have a way to quickly screen for adhesion to endothelial cells – the first barrier that particles encounter when injected intravenously. Screening under static conditions in wells-plates ignores the mechanical forces caused by the flowing blood, which counter particle adhesion. A recent study by our group, using fluorescent siRNA-containing polymer particles, shows that microfluidic technology allows for quick screening of particle adhesion to endothelial cells under dynamic conditions (see Appendix of this thesis).[193] More realistic microfluidic models of microvasculature have been developed by Prabhakarpandian et al.[141] for the same purposes. These devices contain channels that are designed after real capillary networks. They show that capillary geometry has a strong influence on local mechanical conditions and particle

adhesion. The fabrication of a more complex microfluidic device that tries to mimic the tight blood-brain barrier, while still being easy to use in high-throughput assays was recently reported by Genes et al.[52] It is to be expected that high-throughput screening in these more realistic vascular models of the in vivo situation will become the norm in drug development and material science in the future.

Stem cells and tissue engineering

Regenerative medicine is the field in which researchers try to engineer tissues in the laboratory to replace damaged or missing tissue in the human body. It is a multidisciplinary field, which combines materials science with cell biology and biomedicine. A major challenge in this field is the production of vascularized tissue for implantation. In order to achieve this, stem cells must be stimulated to differentiate into vascular cells, and these vascular cells need to arrange themselves into a vascular network. Microfluidic technology can be of use in both processes. For differentiation of human stem cells to vascular tissue, many factors can be of influence. Because human stem cells and the inducing factors are relatively difficult to obtain, it is advantageous to perform tests in a microfluidic setting instead of in a macroscopic assay. Figallo et al.[48] developed a 12-wells micro-bioreactor in which human embryonic stem cells were directed towards a vascular phenotype by varying growth factors, perfusion and cell seeding density. Using such microfluidic devices instead of conventional techniques saves reagents and allows for more flexibility in terms of culture parameters.

The other aspect of vascular tissue engineering in which microfluidic technology can be of help is in preparing vascular networks that can be incorporated into tissue constructs. This can be accomplished by two approaches. First of all, a 'synthetic capillary' can be engineered by using microfluidic technology. This works by designing a microfluidic channel of which the walls contain tiny gaps of only a few micrometer in diameter and tens of micrometers in length. Behind these gaps, compartments are located in which tissue can be grown. When medium is pumped through the channel, the gaps act as a simple endothelium-like barrier, limiting mass transport to the tissue compartments. An example of such a microfluidic design was reported by Lee, et al.,[99] who used this principle to design synthetic analogs of liver sinusoids. Using this approach, mass transport over the endothelium-like barrier can be tweaked to mimic the values found in human vessels.[83, 84] A second approach to use microfluidic technology for generation of vascular networks is based on the notion that the microfluidic device itself can be considered as a three-dimensional 'scaffold' in which cells can be grown. When all sides of a channel are completely covered with endothelial cells, microvascular networks are generated that mimic in vivo networks (figure 6). It has been shown that this approach will in principle work: PDMS

devices with microvascular network morphologies can be completely covered with endothelial cells to generate capillary-like structures.[162] However, PDMS is a non-biodegradable polymer. Biodegradability is paramount if eventually the material is to be replaced with functional tissue. Another study has shown that the same type of system can also be built with the biodegradable polymer poly(glycerol sebacate) or with the biopolymer collagen I.[47, 55] Still, these microfluidic devices consist of only one flat layer of vascular structures. A major challenge will be to build biodegradable, truly three-dimensional microvascular networks that can be combined with other materials and cells in regenerative medicine. Novel rapid techniques for three-dimensional device fabrication, such as stereolithography with biodegradable polymers,[139] hold great promise to overcome this challenge.

Conclusion

The examples given in this review clearly illustrate the fact that the use of microfluidic technology facilitates current vascular research and, more importantly, opens up novel areas of research that are not possible with more conventional set-ups and techniques. It is important to realize that microfluidic technology not only paves the way for more realistic in vitro models in vascular cell biology, but that the technology is still in its infancy in terms of throughput. Almost all studies described in this review are proof-of-principle experiments that require a lot of personal effort and intervention by the researcher. However, automation, standardization and increasing scale will all be natural stages in the maturation of microfluidic technology. These improvements will boost the systematic nature of vascular cell biological research in the future.

Chapter 3: Lowering caveolin-1 expression in human vascular endothelial cells inhibits signal transduction in response to shear stress

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Vascular endothelial cells have an extensive response to physiological levels of shear stress. There is evidence that the protein caveolin-1 is involved in the early phase of this response. In this study, caveolin-1 was downregulated in human endothelial cells by RNAi. When these cells were subjected to a shear stress of 1.5 Pa for 10 minutes, activation of Akt and ERK1/2 was significantly lower than in control cells. Moreover, activation of Akt and ERK1/2 in response to vascular endothelial growth factor was significantly lower in cells with low levels of caveolin-1. However, activation of integrin-mediated signaling during cell adhesion onto fibronectin was not hampered by lowered caveolin-1 levels. In conclusion, caveolin-1 is an essential component in the response of endothelial cells to shear stress. Furthermore, the results suggest that the role of caveolin-1 in this process lies in facilitating efficient VEGFR2-mediated signaling.

Introduction

Vascular endothelial cells (ECs) are constantly subjected to shear stress caused by the flow of blood. ECs are highly responsive to changes in this shear stress. They are able to convert these mechanical stimuli into relevant biological signals by a process that is known as mechanotransduction.[107] The best-known elements in the early stages of this response are the cell-anchoring integrins[78] and certain membrane-associated receptors, such as the vascular endothelial growth factor receptor-2 (VEGFR-2)[79, 187] and G-protein coupled receptors.[57] After the initial activation of these molecules, the biological signal is transmitted into the cell by activation of major signal transduction pathways, such as mitogen-activated protein kinase (MAPK) pathways, the protein kinase B (PKB/Akt) pathway and the endothelial nitric oxide synthase (eNOS) signaling route. These events lead to a functional response of the cell, influencing rate of apoptosis and proliferation,[81, 107] sensitivity to inflammation[161] and cytoskeletal remodeling.[133]

Studies have shown that 50-nanometer, omega-shaped membrane invaginations, known as caveolae, are linked to mechanotransduction. The majority of membrane-associated proteins that are phosphorylated in response to shear stress localize to

these domains[145]. Also, as ECs are subjected to shear stress, the density of caveolae in the cell membrane increases, modulating the activation of signaling pathways.[14, 146] Moreover, mice that lack a structural protein of the caveolar domain, caveolin-1, have an abnormal vascular response when shear stress is altered.[214]

In order to gain more mechanistic insight into the exact role of caveolae in the EC response to shear stress, in vitro studies were carried out to interfere directly with caveolar function. In some studies, caveolae were disrupted by cholesterol extraction[103, 137, 145] and in one study, caveolar functioning was inhibited by introducing blocking antibodies to caveolin-1.[136] These studies have shown that interfering with caveolar function causes an impaired response to shear stress, as characterized by a lowered activation of MAPKs,[136, 137, 145] Akt[103] and eNOS.[145]

An important molecular biological tool to study the role of proteins in cellular processes is RNA interference (RNAi). By transfecting cells with short interfering RNA (siRNA) molecules with a sequence that is complementary to the mRNA of the protein of interest, a dramatic lowering of the expression of this protein can be achieved. The tool is very specific and using it to lower expression levels of caveolin-1 could be useful in confirming the results obtained by the more crude method of cholesterol extraction.

In this study we use RNAi in human umbilical vein endothelial cells (HUVECs) to confirm the essential role of caveolin-1 in EC mechanotransduction. We also show that lowering caveolin-1 levels leads to impaired VEGFR-2 signaling, but not integrin signaling, in these cells. This suggests that the role of caveolin-1 in mechanotransduction lies in coupling VEGFR-2 activation to downstream signaling.

Methods

Antibodies and reagents

Caveolin-1 siRNA was bought from Qiagen (cat. no. SI00299635); negative control siRNA was obtained from Invitrogen (Stealh RNAi negative control with medium GC content). Antibodies for immunodetection were from the following companies: caveolin-1 (Sigma, C4490), Akt (Abcam, ab28422), phospho-Akt (Abcam, ab27773), ERK1 (Abcam, ab9363), phospho-ERK (Santa Cruz, sc 7976), GAPDH (Abcam, ab9485), goat anti-rabbit IgG-Alexa 633 (Molecular Probes, A21071), goat anti-rabbit IgG-HRP (Sigma, A0545), mouse anti-goat IgG-HRP (Zymed, 81-1620). All products for cell culturing were from Lonza, except for the partially purified fibronectin, which was obtained as a coproduct during purification of human factor VIII at Sanquin, Amsterdam, The Netherlands and which was used at 2 mg/ml in phosphate buffered saline (PBS) to coat surfaces for cell culture. All other reagents were from Sigma, except when specified differently.

HUVEC isolation and culturing

HUVECs were isolated from umbilical cords by the method of Jaffe, et al.,[77] using trypsin solution (0.05% (w/v) trypsin, 0.02% (w/v) EDTA in PBS). The obtained endothelial cells were cultured in fibronectin-coated culture flasks in Endothelial Growth Medium-2 (EGM-2), containing 2% fetal bovine serum, until they reached confluency. When confluent, cells were detached from the surface using trypsin solution and diluted 1:3 in a fresh fibronectin-coated culture flask. Cells were kept in a humidified incubator (37°C, 5% CO_2) up to passage 8, after which they were discarded.

siRNA transfection

The day before transfection, HUVECs were seeded in a 6-wells plate, $300 \cdot 10^3$ cells per well in EGM-2. The following day, cells were washed once with OptiMEM medium (Gibco) and then overlaid with 800 µl OptiMEM. To transfect one well with siRNA, the following protocol was used. 15 µl of 20 µM siRNA solution was mixed with 145 µl of 37°C OptiMEM medium and left at room temperature for 15 minutes. 8 µl of Oligofectamine (Invitrogen) was mixed with 32 µl OptiMEM and after 5 minutes the Oligofectamine mixture was added to the tube with siRNA. Complexes were allowed to form for 15 minutes and they were added to the cells. After 4 hours in the incubator, 500 µl EGM-2 with three times the normal amount of bovine serum was added to the wells. After overnight incubation, the cells were washed with PBS and incubated in normal EGM-2 for 6 hours.

Cell treatments

For shear stress experiments, four wells of siRNA transfected cells were trypsinized and replated on a fibronectin-coated glass plate of 40 cm^2. The cells were left to adhere for two hours in EGM-2, after which the medium was replaced by Endothelial Basal Medium-2 (EBM-2) to starve the cells overnight. The next day, 48 hours after transfection, cells were subjected to shear stress in a custom-built parallel plate flow chamber. All parts of the set-up were sterilized before use. The chamber consisted of two parallel glass plates, spaced 0,6 mm apart by glass spacers, held together by a stainless steel housing. The chamber was connected to a glass reservoir containing 200 ml shear medium (Medium 199 with 100 units/ml penicillin and 100 µg/ml streptomycin) and to a peristaltic pump (Watson-Marlow). The different parts of the set-up were connected to each other in a closed circuit using silicone tubing (Versitec silicone, 5 mm inner diameter, Rubber BV, The Netherlands) and the entire set-up was put in an incubator to maintain proper culturing conditions. Medium was then pumped through the flow chamber for 10 or 30 minutes at a rate of 200 ml per minute. This yields a theoretical estimate for the shear stress of approximately 1.5 Pa.

After the treatment, the flow chamber was disassembled, the cells were washed once with PBS and scraped in 100 µl ice-cold lysis buffer (1% (w/v) Triton X-100/PBS with added protease inhibitors (4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin) and phosphatase inhibitors (microcystin LR, cantharidin, (−)-p-bromotetramisole, sodium vanadate, sodium molybdate, sodium tartrate, and imidazole)). Then, the lysate was incubated on ice for 15 minutes and centrifuged at 12000 x g for 10 minutes. The supernatant was transferred to a fresh tube and the protein concentration was determined using a bicinchonic acid assay (Pierce). An equivolume of 2x Laemmli sample buffer was added to the lysate. The resulting sample was boiled for 5 minutes and then stored at -20°C until use.

For $VEGF_{165}$ stimulation experiments, siRNA transfected cells were incubated overnight in growth medium and then starved for 6 hours with EBM-2. Subsequently, 48 hours after transfection, the cells were washed once with PBS, followed by addition of EBM-2 with 200 ng/ml $VEGF_{165}$. Cells were put in the incubator for 10 minutes, after which a lysate was prepared as described above.

The integrin activation experiments were performed as follows. After siRNA transfection, cells were starved overnight in EBM-2. The following day, 48 hours after transfection, cells were trypsinized, spun down and resuspended in 2 ml EBM-2 with 2% (w/v) bovine serum albumin (BSA). The cells were kept in suspension in the incubator under constant, light agitation for 30 minutes. Then, 1 ml of the cell suspension was plated on a fibronectin-coated surface, while the remaining cells were spun down, washed once with PBS and then resuspended in lysis buffer. The plated cells were left to adhere for 20 minutes, after which a lysate was prepared as described above.

Western blot and immunodetection

Samples were subjected to sodium dodecyl sulphate (SDS) poly(acrylamide) gel electrophoresis, Western blotting and immunodetection according to common protocols. Shortly, samples were separated by size on a 10% (w/v) poly(acrylamide) gel and the protein band pattern was transferred to a poly(vinylidene difluoride) membrane, using a Bio-Rad Mini Protean 3 system. The membrane was blocked with 1% (w/v) non-fat dry milk in 25 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 8.3 (TBS-T). The primary antibodies were applied to the membranes in blocking buffer, overnight at 4°C. After washing the membrane with TBS-T four times for 20 seconds and one time for 15 minutes, the secondary antibody was applied to the membrane in blocking buffer for one hour at room temperature. The same washing regime was repeated and the membrane was overlayed with SuperSignal West Femto substrate (Pierce). After three minutes of incubation, the chemiluminescent signal was detected with a Kodak Image station with CCD camera.

Confocal microscopy

Cells were seeded at a density of $150 \cdot 10^3$ cells per well on fibronectin-coated glass coverslips in a 12-wells plate. They were transfected with siRNA as described earlier, but with half the reagents per well. After transfection, the cells were incubated overnight in EGM-2, then washed with PBS and fixed with 4% (w/v) formaldehyde/PBS for 15 minutes at room temperature. The coverslips were then covered with permeabilization buffer (PBS with 1 mg/ml BSA and 0,1% (w/v) Triton X-100) for 10 minutes. Primary antibodies were diluted in permeabilization buffer and then incubated on the coverslips for 1 hour at 37°C. The coverslips were washed three times for 5 minutes with PBS and were subsequently covered with the secondary antibody in a mild permeabilization buffer (PBS with 1 mg/ml BSA and 0,05% (w/v) Triton X-100). After incubating for 1 hour at 37°C, coverslips were washed and a 100 ng/ml solution of 4',6-Diamidino-2-phenylindole (DAPI) was applied for 5 minutes to stain nuclei. After three more washes with PBS, coverslips were mounted on microscope slides using Mowiol (CalBiochem) and stored at 4°C in the dark until they were imaged with a Zeiss LSM 510 confocal microscope. An estimate of the average staining intensity was performed by dividing the total signal in a field by the number of nuclei, as determined by using ImageJ image analysis software.[1]

Statistical analysis

Signal intensities of immunodetection on Western blots were quantified using ImageJ. The intensity of the specific signal was normalized to total protein content, as assessed by the loading control in the same lane. In order to compare these signal intensities between different experiments, the ratios between the intensity level of a sample and the total intensity of all samples in that experiment were determined. The averages of these normalized ratios were plotted, with the error bars representing standard deviation. Differences between these means were tested for statistical significance by performing an unpaired, two-tailed student's t-test. Differences were considered to be statistically significant at p-values smaller than 0.05.

Results

Mechanotransduction in ECs

In order to investigate mechanotransduction in our flow chamber system, HUVECs were subjected to physiologically relevant shear stresses for 10 minutes and 30 minutes. After these treatments, cell lysates were tested for phosphorylated Akt and ERK1/2 by immunoblotting. As is shown in figure 7, 10 minutes of shear stress led to significantly enhanced phosphorylation of both Akt and ERK1/2. After 30 minutes of shear stress, phosphorylation levels had dropped to values comparable to the

static situation. Therefore, we decided to focus on the early timepoint for our studies into the role of caveolin-1 in the EC mechanotransduction.

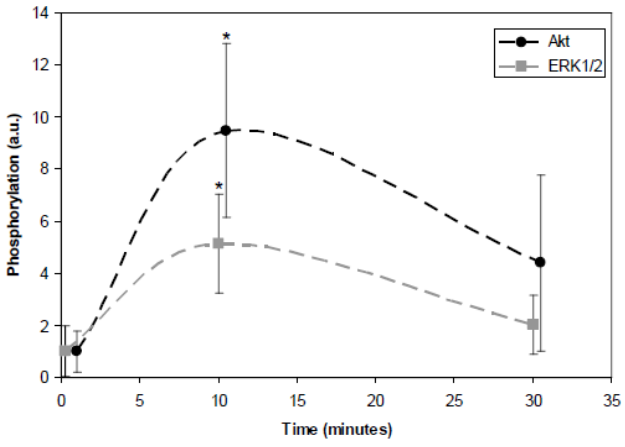

Figure 7: Activation of mechanotransduction pathways by shear. HUVECs were subjected to shear stress for different periods. Subsequently, the amount of phosphorylated Akt and ERK1/2 was determined by immunoblotting. After 10 minutes, phosphorylation levels were significantly higher (* $p < 0.01$, student's t-test) compared to levels in statically cultured cells. After 30 minutes, this significant increase was no longer detected.

Caveolin-1 downregulation

When HUVECs were treated with caveolin-1 siRNA, the expression of caveolin-1 decreased significantly to less than one-third of the caveolin-1 level in untreated cells, as shown by immunoblotting (figure 8a, b). When cells were transfected with negative control siRNA, no statistically significant decrease in caveolin-1 expression was detected, showing the specificity of the siRNA treatment. Moreover, expression of Akt and ERK1/2 was not affected by the transfection procedure or the lowering of caveolin-1 levels (figure 8a). The downregulation of caveolin-1 was confirmed by using immunocytofluorescence (figure 8c). Using the same microscope settings, staining intensity was approximately four times higher in untreated cells than in caveolin-1 siRNA-treated cells. The remaining caveolin-1 in the siRNA-treated cells was located mostly in the perinuclear area, with a lack of intense membrane staining like in the untreated and control RNA treated cells.

Mechanotransduction in ECs with lowered caveolin-1 levels

HUVECs with normal and lowered levels of caveolin-1 were subjected to physiological levels of shear stress in a parallel plate flow chamber. After 10 minutes of shear stress, phosphorylation status of the important mechanotransducing molecules Akt and ERK1/2 was determined by phospho-specific immunoblotting. Total expression levels of these proteins were not affected by siRNA treatment, as determined by immunoblotting (figure 8a). After subjecting HUVECs to shear stress, phosphorylation status of Akt and ERK1/2 increased approximately five to ten times (figure 9). We found that the activation of both signal transduction pathways was significantly lower in caveolin-1 siRNA-treated cells than in cells treated with negative control siRNA. No significant differences were found between the activation levels of control siRNA-treated cells and untreated cells.

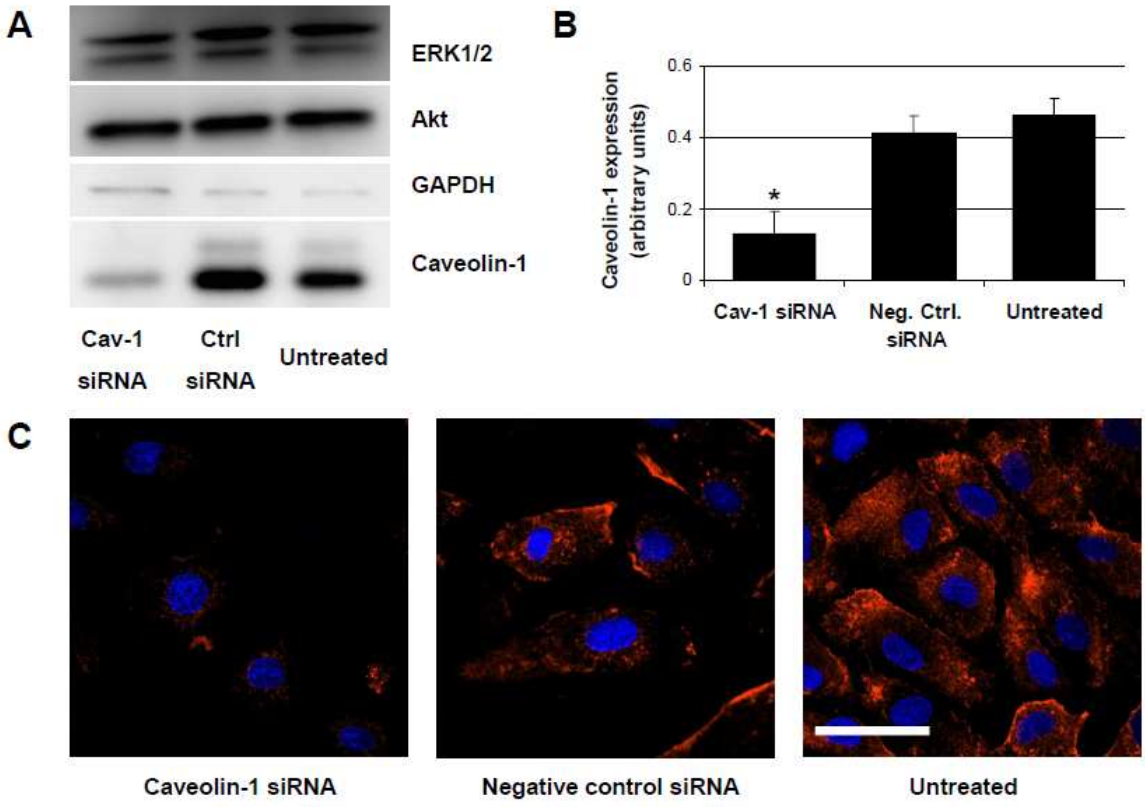

Figure 8: Downregulation of caveolin-1 expression by RNAi. HUVECs were treated with caveolin-1 siRNA, negative control siRNA or left untreated. Caveolin-1 levels were determined 48 hours after transfection. A. Immunoblot analysis of cell lysates. Equal loading of proteins of each transfection condition was ensured by detecting the unrelated metabolic enzyme GAPDH on the same blot. B. Quantification of the signal intensities in the immunoblot assay. The bars represent average intensities of the caveolin-1 band in seven separate experiments. Error bars represent standard deviation. Caveolin-1 siRNA treated cells had significantly lower caveolin-1 expression than control siRNA-treated cells (* $p < 0.0001$, student's t-test). No significant differences were found between untreated cells and negative control siRNA-treated cells. C. Confocal microscopic imaging of caveolin-1. Treated cells were fixed and labeled with caveolin-1 antibodies that were detected by Alexa 633-coupled secondary antibodies. Nuclei were stained with DAPI. All images were taken with the same microscope settings. Scale bar is 50 µm.

Signal transduction in ECs with lowered caveolin-1 levels

In order to uncover the mechanism for the ineffective mechanotransduction in cells with low caveolin-1 levels, we induced signal transduction by activating cell surface receptors. We chose activation of integrins and VEGFR-2, because these proteins are well-known to be essential for mechanotransduction. In order to assess integrin-mediated signal transduction, cells were kept in suspension for 30 minutes and then plated for 20 minutes on a fibronectin-coated surface. When checking the phosphorylation status of Akt and ERK1/2 by phospho-specific immunoblotting, no significant differences could be detected between the activation of signal trans-

duction pathways in negative control siRNA-treated cells and in cells with low caveolin-1 levels (figure 10a, b). Activation of VEGFR-2 was accomplished by stimulating starved cells with 200 ng/ml $VEGF_{165}$ for 10 minutes. In this case, signal transduction was found to be significantly lower in cells that had been treated with caveolin-1 siRNA than in cells with normal levels of caveolin-1 (figure 10c, d).

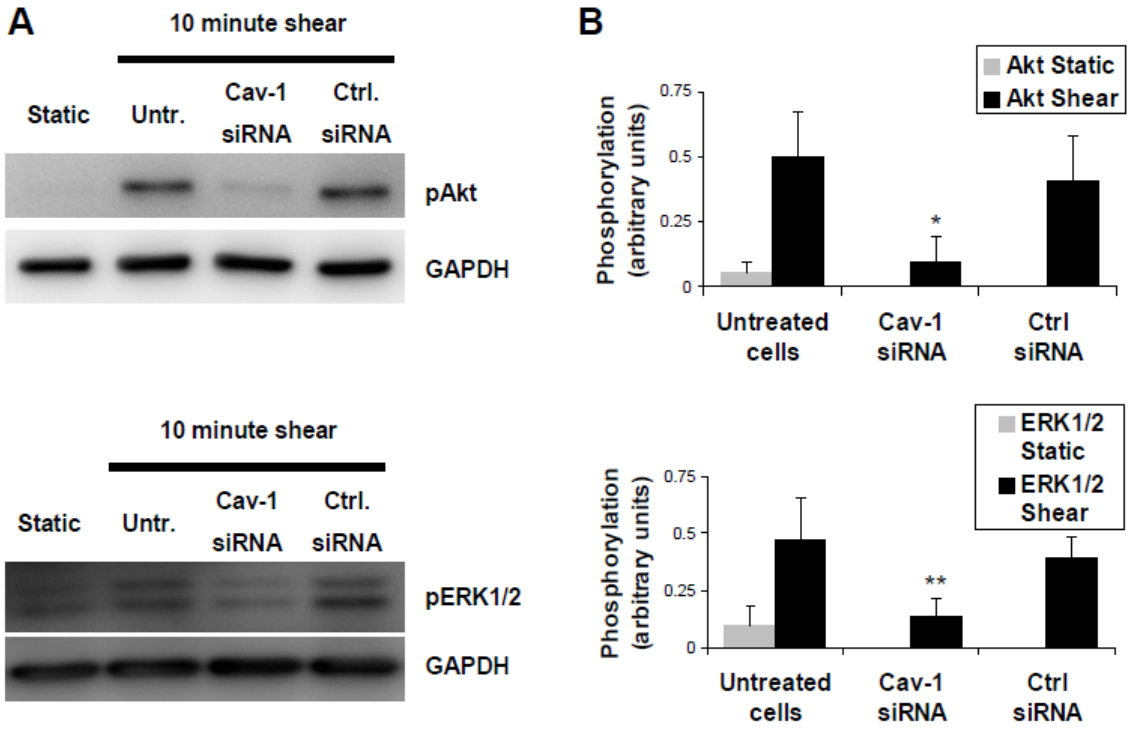

Figure 9: Activation of Akt and ERK1/2 in response to shear stress. HUVECs were transfected with either caveolin-1 siRNA, negative control siRNA or left untreated. 48 hours after transfection, cells were subjected to a shear stress of approximately 1.5 Pa for 10 minutes. A: After treatment, cells were lysed and the amount of phosphorylated Akt and ERK1/2 was determined by immunoblotting. Equal loading of proteins of each transfection condition was ensured by detecting the unrelated metabolic enzyme GAPDH on the same blot. The phosphorylation status of statically cultured cells is shown as a reference. B: Quantification of the signal intensities in the immunoblot assay. Each bar represents the average intensity of four separate experiments. Error bars denote standard deviation. Akt and ERK1/2 phosphorylation increase five- to tenfold when untreated cells are subjected to shear stress. Both Akt and ERK1/2 phosphorylation are significantly lower (* $p < 0.05$, ** $p < 0.01$, student's t-test) when cells are treated with caveolin-1 siRNA instead of negative control siRNA prior to subjecting the cells to shear stress. No significant difference was detected between untreated cells and cells transfected with negative control siRNA, showing that the transfection procedure itself has no influence. Graphs represent the averages of four separate experiments.

Discussion

Our study shows that lowering the expression level of caveolin-1 in human ECs by RNAi inhibits mechanotransduction in response to shear stress. Thus, we confirm

the essential role of caveolae in the EC response to shear stress which has also been suggested by research groups that interfered with caveolar function using methods like cholesterol extraction[137] and antibody blocking.[136]

We show that the activation of both Akt and ERK1/2 is affected by the lowering of caveolin-1. In both cases, this lowering will have functional repercussions for the cells. Akt is well-known for its role in cell survival and resistance to apoptosis. Indeed, activation of Akt in response to shear stress has been shown to suppress apoptosis in ECs.[38, 103] Based on the findings in this study, one would expect that ECs with low caveolin-1 levels are more sensitive to apoptosis when subjected to shear stress than untreated cells. This would be an interesting and counterintuitive result, because lack of caveolin-1 generally renders cells less sensitive to apoptosis.[109] The exact mechanisms of this decreased apoptosis sensitivity have not yet been elucidated, although the Akt pathway is definitely involved.[109] A number of important membrane receptors in ECs, such as activin receptor-like kinase 1 (ALK-1),[153] VEGFR-2[95] and epithelial growth factor receptor (EGFR),[85] have been shown to associate with caveolin-1. Moreover, all mentioned receptors have been shown to induce Akt signaling.[53, 91, 179] Therefore, loss of the association between these receptors and caveolin-1 by downregulation of the latter may be a reason for changes in Akt activation and apoptosis sensitivity. Given the importance of EC apoptosis in the onset and development of atherosclerosis,[147] future studies should be performed to provide more insight in this functional implication of the substantially lowered Akt activation in cells with less caveolin-1.

Another well-known, downstream effect of Akt activation is increased eNOS activity.[39] Based on our finding that lowered levels of caveolin-1 inhibit activation of Akt, it can be expected that eNOS activation and the resulting effects on blood vessels, such as vasodilatation, are also impaired. Ex vivo studies have indeed shown that eNOS activation and vasodilatation in response to fluid flow are lower in arteries of caveolin-1 knockout mice than in blood vessels of wild-type mice.[214]

The dramatically lowered activation of ERK1/2 in response to shear stress in caveolin-1 siRNA-treated ECs that we describe in this study will also have functional implications. Activation of ERK1/2 has an important role in modulating gene expression in response to shear stress. Two well-described examples show that ERK1/2 activation is essential for the shear stress-induced increase in the expression of matrix metalloprotease-9[174] and eNOS.[34] The absence of ERK1/2 activation in our system will surely have an impact on these shear stress-induced changes in expression.

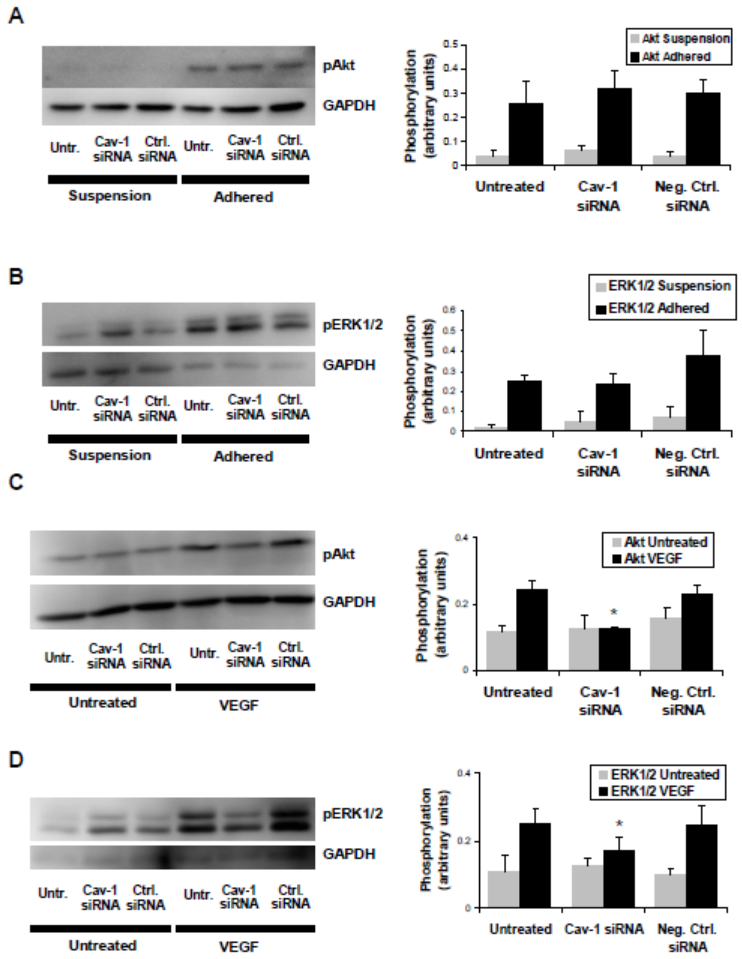

Figure 10: Activation of Akt and ERK1/2 in response to external stimuli. HUVECs were transfected with either caveolin-1 siRNA, negative control siRNA or left untreated. 48 hours after transfection, cells were subjected to the following stimuli. A-B: Cells were kept in suspension for 30 minutes and then plated on fibronectin-coated culture plates to activate integrin-mediated signaling. Phosphorylation of both Akt (A) and ERK1/2 (B) increased significantly when compared to cells in suspension. No significant differences in activation levels were detected between HUVECs with high or low caveolin-1 levels. C-D: Cells were starved in basal medium and then treated with 200 ng/ml VEGF for 10 minutes. Phosphorylation of both Akt (C) and ERK1/2 (D) in response to this stimulus was significantly lower in cells with low levels of caveolin-1 than in cells that were treated with negative control siRNA (* $p < 0.01$). All bars are averages of three separate experiments.

When attempting to identify the mechanism by which caveolin-1 contributes to mechanotransduction, we first decided to investigate the signal transduction in response to integrin binding in these cells. Integrin-mediated signaling is an essential process in mechanotransduction.[12, 78] Moreover, there have been multiple reports of the involvement of caveolin-1 in this signaling pathway.[142, 202, 203] Despite these reports, we were unable to detect any changes in activation of Akt or ERK1/2 between caveolin-1 siRNA-treated cells and untreated cells. It could be that in our system the remaining caveolin-1 is still sufficient to properly induce these signaling events. Our system differs from the ones used in the aforementioned reports by cell type, caveolin-1 levels and the chosen output parameters to determine efficient signaling. Based on our experiments we can conclude that it is unlikely that the inefficient mechanotransduction as seen in our system is due to impaired integrin signaling.

We decided to also investigate signal transduction in response to $VEGF_{165}$ in caveolin-1 siRNA-treated ECs. Ligand-independent activation of VEGFR-2 is another essential event in mechanotransduction[79] and stimulation with $VEGF_{165}$ can serve as a way to specifically activate and monitor this part of the signaling network. The role of caveolin-1 in $VEGF_{165}$-induced signal transduction is still ambiguous and may depend on cell type and culture conditions. In some cell systems, downregulation of caveolin-1 leads to hyperactivation of signal transduction pathways.[56] In addition, other studies that focus on the role of caveolin-1 in $VEGF_{165}$-initiated signaling have shown that both an increase and a decrease of the levels of caveolin-1 in ECs have a negative impact on downstream signal transduction.[95, 169] Caveolin-1 is thought to be inhibitory for VEGFR-2 activation by directly associating with it, but this association also seems necessary for proper initiation of downstream signaling after growth factor receptor activation. Moreover, caveolin-1 knock-out mice have impaired vasculogenic potential, due to impaired $VEGF_{165}$ signaling.[169] Also in our system, lowering the level of caveolin-1 has a negative impact on signal transduction when ECs are stimulated with $VEGF_{165}$. This suggests that the lack of mechanotransduction in ECs with low caveolin-1 levels is due to the important role that caveolin-1 plays in coupling VEGFR-2 activation to downstream signaling events.

Based on the results presented in this article, it can be concluded that caveolin-1 is needed for activation of Akt and ERK1/2 in ECs that are subjected to shear stress. Our results suggest that the impaired activation of these pathways in ECs with low levels of caveolin-1 is due to inefficient VEGFR-2 signaling. This study highlights the importance of caveolin-1 in normal EC functioning. The role that caveolin-1 plays in the biology of ECs and the impact it has on the physiology of the vasculature in vivo are becoming more and more clear. Because of the importance of caveolin-1 in endothelial signaling, it is a potential target for clinical applications, e.g. in tumor angiogenesis and atherosclerosis. Therefore, the role of caveolin-1 in the EC shear stress response is definitely worth studying in more detail.

Chapter 4: Analyzing shear stress-induced alignment of actin filaments in endothelial cells with a microfluidic assay

Manuscript under review for publication: A.D. van der Meer, A.A. Poot, J. Feijen, I. Vermes

Vascular endothelial cell physiology is affected by numerous biochemical and physical factors, one of which is the shear stress that is exerted on the cells by flowing blood. Microfluidic assays are potentially very useful for generating insight into the cell biological processes in the endothelial response to shear stress. In this study, a microfluidic assay was employed for tracking the alignment of actin filaments in response to shear stress. Endothelial cells were cultured and subjected to shear stress in microfluidic channels. Subsequently, actin filaments were stained and their directionality was analyzed by a method depending on transformation of micrographs into the frequency domain. When endothelial cells were subjected to an average shear stress of 1.5 Pa for 16 hours, the alignment of actin filaments in the channel direction increased threefold compared to static cultures. This increase in alignment was significantly lower when the cells were simultaneously treated with inhibitors of the Rho-associated kinase p160ROCK, or of the vascular endothelial growth factor receptor-2 (VEGFR-2). In contrast, inhibition of phosphoinositide 3-kinase (PI3K) had no effect on actin filament alignment. The importance of VEGFR-2 and p160ROCK for cytoskeletal remodeling in response to shear stress has been described previously. The results in this study now show that the PI3K pathway is not needed in this process. This study is the first to employ a microfluidic assay for generating mechanistic insight into shear stress-induced processes in endothelial cells. It may serve as an example for employing microfluidic technology in the field of vascular biology.

Introduction

Vascular endothelial cells form the inner lining of all blood vessels. These cells have an important role in maintaining vascular tone, regulating transport over the vessel wall, attracting white blood cells to sites of inflammation and the formation of new blood vessels.[24] The endothelial physiology is affected by numerous chemical, biological and physical factors. One of these factors is the fluid shear stress that is exerted on the endothelium by the blood flowing over its surface. Endothelial cells show a varied response when they are subjected to shear stress. Gene expression is altered, vasodilatants are released, cell migration is enhanced and apoptosis is inhibited.[107] The most striking change in the endothelial cell response to shear stress is the

change in morphology, which is accompanied by extensive cytoskeletal remodeling. When the shear stress has a clear direction, endothelial cells elongate and orient in the direction of the flow. Moreover, the microtubule organizing center positions itself on the upstream side of the cell and bundles of actin filaments appear in the cell body, parallel to the shear stress direction.

Because vascular disease is predominantly localized to regions with an abnormal mechanical environment, e.g. curves and bifurcations with irregular blood flow patterns,[30] the effects of shear stress on endothelial cells are widely studied. A lot of research is dedicated to identifying the intracellular pathways involved in sensing and transducing the shear stress signal, as well as factors that translate the signal into a functional cellular response. In order to identify these factors, in vitro assays are essential.

There are two main types of set-up for studying the endothelial response to shear stress in the laboratory: the spinning disk device and the parallel plate flow chamber. The former type of set up consists of a rotating disk and a parallel, fixed surface with the cells cultured on it.[51, 68] The disk is brought in close proximity to the surface and the moving fluid between the disk and the plate exerts a shear stress on the cultured cells. Because the tangential velocity of the disk is higher on the edges, the resulting shear stress on the cells also increases away from the centre of the disk. This problem can be overcome by replacing the disk with a shallow inverted cone.[67] In this case, the effect of the difference in velocity is cancelled out by the increasing distance between rotating and fixed surface towards the edges. Thus, a uniform shear stress field can be generated. If the cone and the plate are made of transparent material, these devices can even be combined with microscopy.[158]

The parallel plate flow chamber consists of two transparent plates in close proximity to each other. Medium is pumped through the space between the plates, exerting a shear stress on the cells that are grown on one of the plates.[89, 201] This set-up is easy to combine with microscopy, because the plates are usually made of glass or polystyrene. Also, it is easy to build, because the flow can be generated with any peristaltic or syringe pump. Recently, microfluidic versions of the parallel plate flow chamber have been reported.[20, 160, 168, 181] Micrometer-sized channels were produced with soft lithography. Endothelial cells were cultured inside these channels and were subsequently subjected to physiological levels of fluid shear stress. The use of microfluidic technology in constructing a parallel plate flow chamber offers a number of potential advantages over the conventional macroscopic set-ups. For example, smaller amounts of cells and reagents are needed per experiment. Also, low flow rates can be used to exert physiological levels of shear stress; shear stress is inversely proportional to the third power of the channel dimensions. Another potential advantage is that microfluidic devices can incorporate multiple parallel channels for screen-

ing the effect of a number of inhibitors or for applying a range of different shear stress magnitudes. A related potential advantage is the ease of generating gradients of stimulants or inhibitors in microfluidic devices.[82] This feature can be used to quickly generate dose-response curves. A final advantage of using microfluidics is that it opens up the possibility for incorporating the flow chambers into devices that perform subsequent molecular analysis on the treated samples: the lab-on-a-chip concept.[44]

A number of these advantages have been shown in practice by the groups that developed the aforementioned microfluidic versions of the parallel plate flow chamber. In all of the studies, it is obvious that the amounts of cells and reagents that are needed are significantly lower than in conventional assays.[20, 160, 168, 181] Also, some studies were performed with multiple parallel channels, each of which could be used to treat cells with varying levels of shear stress.[20, 168, 181] Therefore, these studies definitely demonstrate the usefulness of using a microfluidic platform when studying the response of cells to fluid shear stress. Still, the biological insight that is generated by these studies is limited. This is not due to the use of microfluidics in applying shear stress, but because the treatment variables and the monitored output parameters were limited. In all studies, the only treatment variable was the level of shear stress; no stimulants or inhibitors were used. The output parameters that were analyzed were cell orientation and shape,[20, 160, 168] monolayer permeability[160] and secretion of von Willebrand factor (vWF).[20] Analysis of cell shape is an indirect way of monitoring cytoskeletal rearrangements. Studying the permeability of an endothelial cell monolayer is relevant for understanding the pathophysiology of a number of permeability-related vascular diseases, such edema or atherosclerosis. The secretion of von Willebrand factor is a physiologically relevant parameter, since this factor is involved in thrombus formation. In summary, these microfluidic studies showed that cell elongation, monolayer permeability and vWF secretion increase when treating endothelial cells with increasing levels of shear stress. All these findings are well-established facts in vascular scientific literature.[50, 107] This description of known effects does confirm the validity of using a microfluidic approach, but it does little to increase cell biological understanding.

In order for microfluidic assays to be useful in unraveling the response of endothelial cells to shear stress, it should be possible to detect changes in cell behavior while treating with bio-active stimulants or inhibitors. In this study, we show an example of this approach. We investigate which upstream pathways are important for the formation of aligned actin filaments in response to shear stress. We focus on inhibition of proteins that are presumed to become activated in different stages of this process. Firstly, vascular endothelial growth factor receptor-2 (VEGFR-2), which is activated within seconds after the initiation of shear stress and is part of an essential

mechanosensory complex.[187] Secondly, phosphoinositide 3-kinase (PI3K), which is a component of the protein kinase B (PKB/Akt) pathway. This is one of the major signal transduction pathways that become activated in response to shear stress.[38] Finally, Rho-associated coiled-coil forming protein serine/threonine kinase (p160ROCK), which is an important downstream target of the small GTPase Rho. The Rho/p160ROCK pathway is known to be involved in the formation of actin fibers and focal adhesions in response to shear stress.[105]

We perform this study by seeding and culturing endothelial cells in microfluidic channels and subjecting them to shear stress in the absence or presence of the inhibitors. We use a fast, automated image analysis method to measure alignment of actin filaments as an output parameter. Our study is an example of how biological insight can be generated when using a microfluidic shear stress assay. It will be interesting to combine this type of approach with the beautiful microfluidic engineering feats in the aforementioned studies and in the field of microfluidics in general. This combination will be very useful for generating new insight into the endothelial response to shear stress.

Methods

Materials

Polydimethylsiloxane (PDMS, Sylgard 184) for device preparation was from Dow Corning, USA. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by the protocol described by Jaffe.[77] Cell culture media and additives were all from Lonza Benelux BV, except for OptiMEM medium, which was obtained from Invitrogen, USA. Partially purified fibronectin was obtained as a coproduct during purification of human factor VIII at Sanquin, Amsterdam, The Netherlands. The various inhibitors were from Sigma, Germany (Y-27632, LY294002) and CalBioChem, USA (VEGFR-2 Kinase Inhibitor). Phalloidin-FITC was from Sigma, Germany. The Live/Dead Cell Viability assay and 4',6-Diamidino-2-phenylindole (DAPI) were purchased from Invitrogen, USA. The syringe pump was from Sage Instruments, USA, 8 mm inner diameter Tygon tubing from Rubber BV, The Netherlands and blunt 18 gauge needles were from EFD, Inc., USA.

Device fabrication

Microfluidic channels were prepared by pouring PDMS onto a silicon wafer with rectangular SU-8 photoresist structures (100 μm wide, 120 μm high, 2 centimeters long). After crosslinking at 60°C for two hours, PDMS was peeled off of the wafer and holes were punctured in the PDMS to reach the channel. Subsequently, the surfaces of the PDMS devices and 50 mm diameter glass coverslips were activated by

treatment in an oxygen plasma sterilizer at 100 W for one minute. Irreversible bonding between the two surfaces was accomplished by pressing them together.

Cell culture

HUVECs were kept in culture with Endothelial Growth Medium-2 (EGM-2) in 2 mg/ml fibronectin-coated polystyrene flasks. Every other day, cells reached confluence and were trypsinized and replated in fresh flasks. Cells were used in experiments from passage two to seven. When seeding cells in microfluidic channels, cells were spun down (300 × g, 5 minutes) and resuspended to reach a concentration of $20 \cdot 10^6$ cells/ml. This cell suspension was then pipetted into a fibronectin-coated channel. The device was overlaid with medium to limit evaporation and then incubated for six hours in a humidified incubator at 37°C, 5% CO_2. After incubation, channels were flushed once to remove any non-attached cells and confluency was checked by phase-contrast microscopy. Cell viability was checked by incubating the cells with a 1:1000 dilution of the two components of a Live/Dead cell viability assay for 30 minutes. In this assay, viable cells are stained with green fluorescent calcein, while the DNA of dead cells is labeled with red fluorescent ethidium homodimer-1. These fluorescent labels were visualized on a Zeiss LSM 510 confocal laser scanning microscope with a 40× objective.

Shear stress application

When performing a shear stress experiment, the endothelial cell-covered microfluidic channels were connected to a syringe pump using Tygon tubing and blunt needle connections. The shear stress medium was Medium 199, containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and, in some experiments, a specific inhibitor. The medium was pumped through the channels at a rate of 1.5 ml/hour for different time periods. The average wall shear stress can be calculated by using the following equation: $\tau = 6Q\mu/w^2h$, with τ the shear stress in Pa, Q the flow rate in m^3/s, μ the dynamic viscosity in Pa·s, w and h the width and height of the channel in m. The dynamic viscosity of the medium is approximately the same as that of water at 37°C and was set at $7 \cdot 10^{-4}$ Pa·s. For a flow rate of 1.5 ml/hour, the resulting average shear stress on the wall is approximately 1.5 Pa. However, because the aspect ratio of the channel is nearly one, the shear stress profile in the plane of the cells is parabolic. Cells in the middle of the channel are subjected to a shear stress that is approximately one and a half times higher than the average wall shear stress, while cells that are close to the wall feel a shear stress that is approximately two times lower than the average.[17] The shear stress magnitude of 1.5 Pa is in the upper range of physiological values. This high value was picked to limit cell to cell variations in the shear stress response (as reviewed by Davies, et al.[31]). The small

sample sizes of microfluidic assays make them particularly sensitive to experimental artifacts that are caused by cellular heterogeneities. Stimulating cells with a high shear stress lowers the risk of inducing large variations among cellular responses. All shear stress experiments were performed in an incubator at 37°C and 5% CO_2.

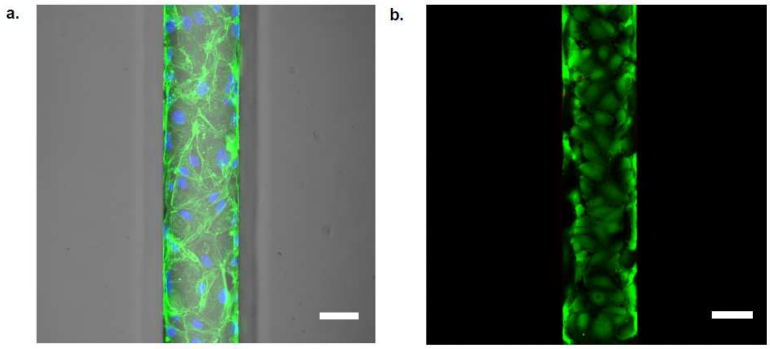

Figure 11: Culturing endothelial cells in a microfluidic device. Human endothelial cells were seeded in a 100 µm wide microfluidic channel and allowed to adhere for 6 hours. a.) Cell morphology was determined by fixing the cells and staining the actin filaments (green) and nuclei (blue). The cells exhibit a well-spread morphology and form a confluent layer in the channel. b.) Cell viability was determined by a live/dead assay, in which living cells are stained green and DNA of dead cells is labeled red. All cells in the microfluidic channel were green fluorescent, demonstrating their viability. Scale bars, 50 µm.

$VEGF_{165}$ treatment

Cells were seeded on chamber slides (Thermo Fisher Scientific, Germany) at densities of either $1{\cdot}10^4$ cells/cm^2 or $3{\cdot}10^3$ cells/cm^2 and left to adhere and grow overnight in EGM-2. The following day, the medium was replaced with EBM-2 containing 10 mg/ml bovine serum albumin (BSA) and the cells were starved for 2 hours. After starvation, some wells were treated with EBM-2, containing 2 mg/ml BSA and 5 µg/ml LY294002 for one hour. Then, the cells were incubated for one hour in the absence or presence of 10 ng/ml vascular endothelial growth factor ($VEGF_{165}$). After this incubation, cells were fixed with 4% paraformaldehyde for 15 minutes. Subsequently, actin filaments were stained by incubating the cells for 30 minutes at 37°C with 1 µg/ml phalloidin-FITC in a buffered salt solution containing 0.1% Triton X-100 and 1 mg/ml BSA. After flushing the cells three times with saline solution, the actin filaments were imaged using confocal microscopy.

Actin filament analysis

After shear stress application, microfluidic channels were detached from the syringe pump and were flushed with 4% paraformaldehyde in saline solution for 15 minutes to fix the cells. Then, actin filaments were stained as described in the previous paragraph and the samples were analyzed with confocal microscopy. For each channel, images of ten different locations were taken. All analysis of actin filament direction was performed with NIH ImageJ image analysis software,[1] using a method

that has been used before for determining directionality in collagen fibers.[21, 128, 197] To our knowledge, this is the first time this method is used to quantify degree of alignment of actin filaments, as well as the first time it is used in a microfluidic assay. The method works by considering the fluorescent micrograph as an interference pattern of intensity waveforms with different amplitude, frequency and direction (see also figure 12 for visualization). If the fluorescent micrograph contains a lot of structures with a specific direction, then there must be an over-representation of high amplitude intensity waveforms with the same direction.

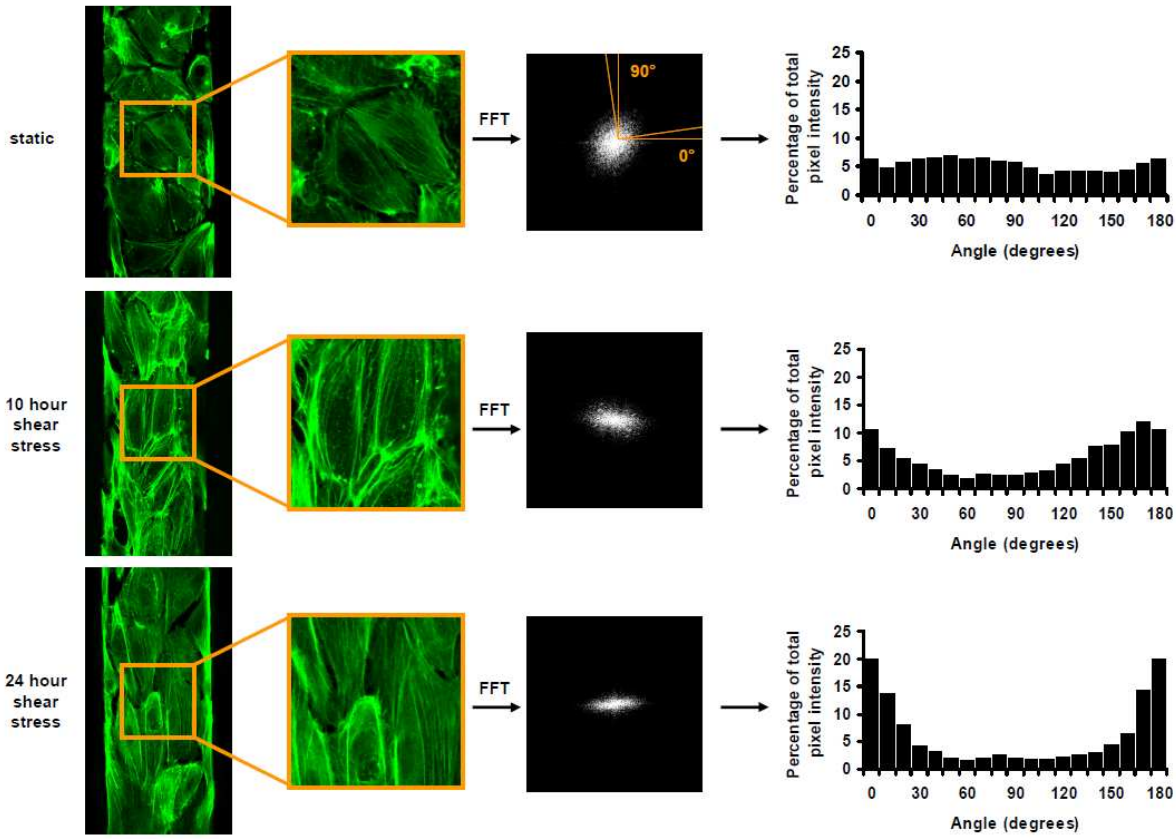

Figure 12: Method for determining actin filament directionality by frequency domain analysis. Endothelial cells were cultured in microfluidic channels, treated with 1.5 Pa shear stress for several periods and then fixed and stained for actin filaments (left). Subsequently, 65 × 65 µm image cut-outs were taken from the middle of the channel to perform the directionality analysis. These cut-outs were automatically transformed to the frequency domain by performing a fast Fourier transform (FFT). The actin filament structures in the original picture can be considered as a pattern of interfering waves of different intensity, frequency and direction. In the frequency domain, the image is decomposed into these directional waveforms. Each pixel in the frequency domain image represents a single wave of color intensity in the original, spatial image. By analyzing the intensity in each 10-degree category of the frequency transform of the image, an angle histogram of waveform direction can be plotted (right). The higher the peak in this histogram, the more aligned waveforms there are in the frequency transform. This is a measure for the number of aligned structures in the original, spatial image.

So, if there would be a way to decompose the original image into the individual waveforms that make up the interference pattern, directional information about the original micrograph could be obtained by simply counting the waveforms with high amplitude and a certain direction. For both the decomposition of the original picture into individual waveforms and the counting of individual waveforms, automated functions exist in ImageJ. First of all, by performing a fast Fourier transform (FFT) on the micrograph, the image is decomposed into individual waveforms, or, technically speaking, transformed from the spatial domain to the frequency domain. The frequency domain version of the micrograph is an image in which each pixel represents an individual waveform. The intensity of a pixel is the amplitude of the waveform it represents, the distance of a pixel to the center of the image gives the frequency of the waveform it represents (low frequency waveforms are closer to the center than high frequency waveforms) and the orientation of the pixel with respect to the center of the image gives the direction of the waveform it represents. So, by analyzing the pixel intensity for each direction in the frequency domain image, information on the directionality of structures in the original micrograph can be deduced.

In practice, the procedure was carried out as follows. First, a 65 μm × 65 μm region was selected from the middle of the channel to limit wall effects. The resulting image was transformed from the spatial domain to the frequency domain by performing an FFT. Waveforms with low amplitude and high frequency represent the fine details in the spatial domain and should therefore be excluded in an analysis of global directionality of fibers. Therefore, the transformed image was contrasted to exclude all low intensity, high frequency signal from the frequency domain image. The remaining high amplitude, low frequency waveforms represent the general structures in the spatial domain. Total pixel intensities for eighteen 10 degree categories in the frequency domain image were calculated. The resulting values were normalized to percentages of total pixel intensity. This procedure was repeated for all ten images of a sample and the percentages for each 10 degree category were averaged. Because of the wall effects in the narrow channels, there is already a slight preference for actin filament alignment in the direction of the channel. This means that the peak values of the averaged histograms were always found in the 0° - 10° or 170° - 180° categories. The percentage of pixel intensity in the 30° peak window consisting of the peak category and the two adjacent categories was used as a measure for the degree of alignment. For easier interpretation, an alignment index was formulated. This index is a value between zero and one. It is calculated by subtracting the percentage of pixel intensity that is expected to be in a 30° window in unaligned samples from the actual percentage of pixel intensity in the 30° peak window and normalizing the resulting value to 1. So, the index is zero when the percentage of pixel intensity in

the 30° peak window is 16.67%, corresponding to an even distribution of pixel intensities over all eighteen 10-degree categories. The index equals one when the total pixel intensity is found exclusively in the 30° peak window. The alignment indexes were compared with data obtained from manual tracking of actin fibers. Manual tracking was carried out by thresholding the fluorescent micrographs to only visualize bright fibers, drawing lines over these prominent structures and measuring the angle of at least 50 lines. Based on these angles, an alignment index was calculated in the same way as described for the frequency domain-based method. This method of manual tracking was also performed with NIH ImageJ software.

Statistical testing

All statistical testing was performed using Microsoft Excel. For testing the significance of applying shear stress or of adding inhibitors during application of shear stress, a student's t-test was performed. When the p-value was smaller than 0.05, the differences between treatment and control were considered significant.

Results

The total volume of medium in a microfluidic channel is only a few hundred nanoliters. When culturing cells inside such a channel, the concentration of nutrients and waste products can shift to values that compromise cell viability within a number of hours.[112] We wanted to perform the experiments on a confluent layer of well-attached and viable endothelial cells. In order to investigate this, the cells were fixed 6 hours after seeding and the actin filaments and nuclei were fluorescently labeled with phalloidin FITC and DAPI, respectively. It was found that the cells covered the entire surface of the channel and exhibited a well-spread morphology (figure 11a). Moreover, we investigated cell viability after 6 hours of culturing without refreshing the medium. This was performed using a live/dead assay, in which viable cells are labeled with green fluorescent calcein and dead cells with red fluorescent ethidium homodimer 1. All cells in the channel were found to be green fluorescent, showing their viability (figure 11b).

Next, we subjected the endothelial cells to shear stress for different periods in order to study the changes in actin filament alignment. When cells were cultured under static conditions in the microfluidic channels for 6 hours or for 24 hours (figure 13a), analysis of directionality with our automated method showed that these samples exhibited an average alignment index of 0.06. When shear stress was applied after 6 hours of static culturing, the alignment index increased linearly to an average value of 0.24 after 24 hours (figure 13a). The increase of the alignment index was accompanied by a visual increase of actin fiber formation in the direction of the flow (figure 12). For the rest of our studies, we always used a shear stress exposure time of 16

hours. At this time point, the degree of alignment has increased up to a point that it is easily discernible from the degree of alignment under static conditions. Moreover, the alignment index has not yet reached a saturated maximum. This will improve the sensitivity for detecting changes in the degree of alignment caused by treatment with inhibitors.

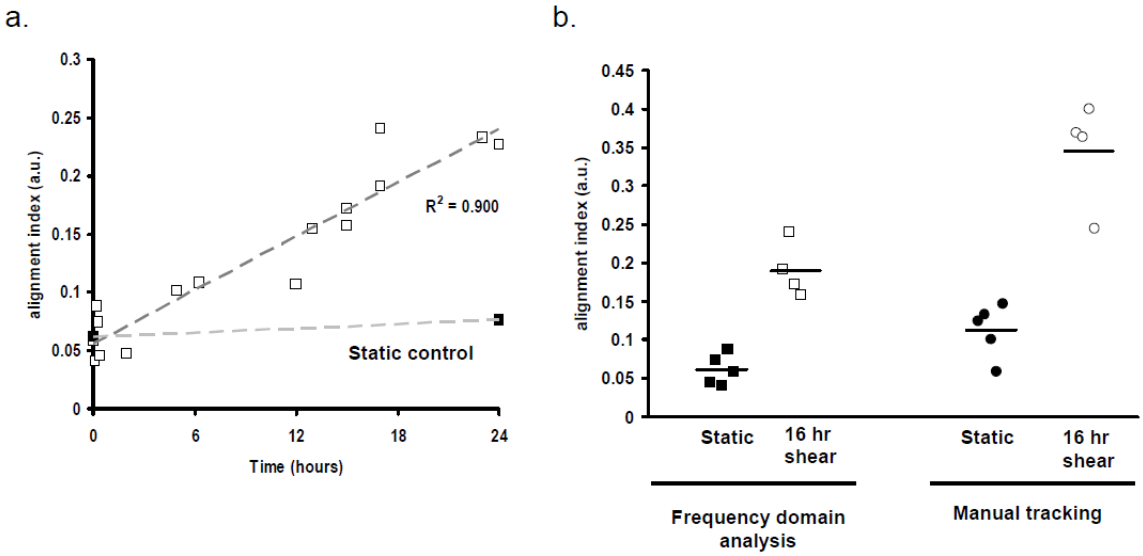

Figure 13: Alignment indexes of statically cultured and sheared samples, as calculated with two different methods. a.) Cells were subjected to a shear stress of 1.5 Pa for different time periods, fixed, stained for actin filaments and analyzed for degree of alignment by frequency domain analysis. Over the time course of 24 hours, the degree of alignment increases almost linearly (open squares and their linear fit). When cells were cultured under static conditions, degree of alignment did not change significantly after 24 hours (filled squares and their linear fit). Based on this data, an exposure time of 16 hours was chosen for all further experiments: at this time point, the degree of alignment is significantly higher than under static conditions, but has not yet reached a maximum. b.) The experimental data was analyzed both by frequency domain analysis (left) and by manual tracking of actin filaments (right). In both cases, samples that were exposed to a shear stress of 1.5 Pa for 16 hours (open squares and circles) were found to have a consistently higher alignment index than statically cultured samples (filled squares and circles). The method of manual tracking produces higher alignment indexes in both statically cultured and sheared samples. Even though the alignment indexes differ depending on the method of analysis, the threefold increase from statically cultured samples to sheared samples is the same in both cases. Each data point represents the alignment index of an independent experiment. Horizontal bars denote the average of each condition.

In order to proof the validity of our method for measuring actin filament alignment, we compared the data obtained by frequency domain analysis with manual tracking of actin fibers. When tracking individual fibers manually and calculating an alignment index, the values were higher than when using our automated method, both under static conditions and after a 16 hour exposure to shear stress (figure 13b). However, both methods of analysis yielded a threefold increase in alignment index from static culture to sheared cells. This shows that our fast, automated method of

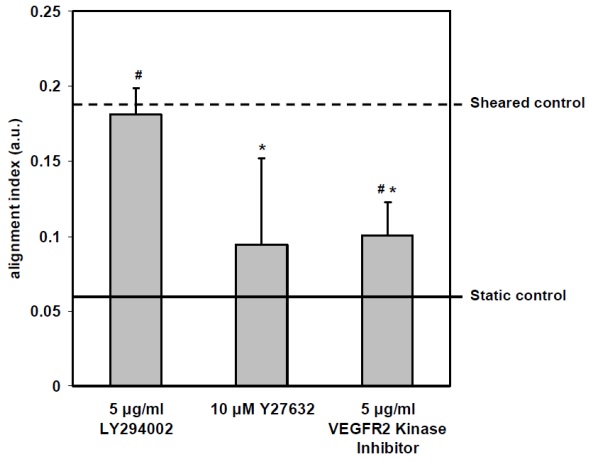

Figure 14: The effect of specific inhibitors on the alignment index in sheared samples. Endothelial cells were treated with 1.5 Pa shear stress for 16 hours in the presence of specific inhibitors of PI3K (LY294002), p160ROCK (Y27632) and VEGFR2 (VEGFR2 Kinase Inhibitor). The dashed line represents the average alignment index of sheared samples without the inhibitors. The solid line denotes the alignment index in statically cultured cells. The bars are an average of three independent experiments. Error bars indicate the standard deviation. Asterisks denote a statistically significant difference from sheared control samples (student's t-test, $p < 0.05$). Hashes denote a statistically significant difference from static controls (student's t-test, $p < 0.05$).

frequency domain analysis of directionality performs equally well as the time-consuming method that relies on manual tracking of fibers.

Using this automated way of quantifying directionality, the effect of several inhibitors on the shear stress-induced cytoskeletal rearrangements was tested. The inhibitors were added to the shearing medium and actin filament directionality was analyzed after 16 hours. As can be seen in figure 14, the inhibitors had different effects on the alignment of actin filaments. Presence of inhibitors of p160ROCK (10 µM Y-27632) or VEGFR-2 (5 µg/ml VEGFR-2 Kinase Inhibitor) led to significantly less shear stress-induced alignment. In contrast, inhibition of the PI3K/Akt pathway by 5 µg/ml LY294002 had no significant effect on alignment.

The absence of an effect of 5 µg/ml LY294002 treatment could also be caused by a lack of inhibitor activity. Therefore, the same inhibitor was tested for its capacity to inhibit $VEGF_{165}$-induced stress fiber formation. The formation of stress fibers by endothelial cells in response to treatment with $VEGF_{165}$ is a well-described, PI3K/Akt-dependent process.[125] As shown in figure 15, when endothelial cells were treated with 10 ng/ml $VEGF_{165}$ for one hour, thick bundles of actin filaments were formed in the cell body. The same treatment did not induce the formation of these stress fibers when the cells had been pre-treated with 5 µg/ml LY294002 for one hour. This shows that the inhibitor does have an effect on cytoskeletal remodeling in the same cell system and at the same concentration, but in response to a different stimulus.

Discussion

In this study, the cytoskeletal rearrangements in endothelial cells in response to shear stress were analyzed. These rearrangements do not simply induce passive changes of cell shape, but they have relevance for vascular physiology. Research has

shown that cytoskeletal dynamics are important for regulating the endothelial barrier function.[13, 122] Cytoskeletal contraction can increase vascular permeability by opening the junctions between endothelial cells. This process is involved in the pathophysiology of acute lung injury and atherosclerosis.[41, 177]

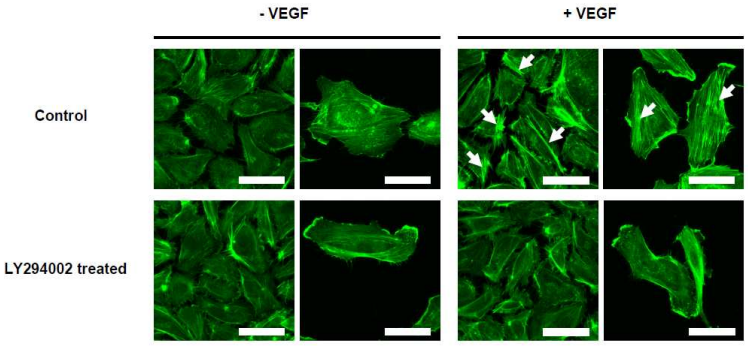

Figure 15: The effect of VEGF treatment and inhibition of the PI3K/Akt pathway on stress fiber formation. In the absence of VEGF, endothelial cells have thin actin filaments running through the cell body (top left). When treated for one hour with 10 ng/ml VEGF (top right), these fibers increase in number and thickness. This effect is absent in cells that have been pre-treated with an inhibitor for PI3K (bottom). Arrows denote the location of thick actin filament bundles. Scale bars, 50 μm.

We used a microfluidic assay to assess the effect of inhibitors on the endothelial cystoskeletal remodeling in response to fluid shear stress. To follow the cytoskeletal remodeling, a fast and automated image analysis method was used. We show that this method performs just as well as manual tracking of stress fibers. By determining actin rearrangements in the presence of both shear stress and inhibitors, we are able to draw biologically relevant conclusions.

Small GTPases, such as Rho and Rac, are the mediators for cytoskeletal remodeling in response to various stimuli, including shear stress.[184] p160ROCK is a downstream target of Rho and is responsible for enhancing myosin light chain activation. Myosin light chain is a component of the filamentous actin network and its activation enhances the contractility of this network and is responsible for the formation of actin stress fibres.[184] Interestingly, endothelial cells that are negative for Rho do not align in the direction of shear stress.[105] However, constant activation of Rho also prevents alignment in the direction of shear stress.[186] Thus, the activation status of the Rho/p160ROCK pathway needs to be finely balanced for the correct cytoskeletal response to take place. In this study, we confirm the need for p160ROCK activation for the efficient alignment of actin filaments in response to shear stress. The Rho/p160ROCK pathway can be considered as a downstream effector pathway in the shear stress-induced cytoskeletal remodeling.

VEGFR-2 is part of a mechanosensory complex that also contains platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and vascular endothelial (VE)-cadherin. Cells that lack one of the components of this complex do not align in the direction of flow.[187] In this study, it was confirmed that VEGFR-2 is essential for cytoskeletal remodeling in response to shear stress. However, which pathways mediate

the effect that activation of VEGFR-2 has on cytoskeletal remodeling is still unclear. A logical candidate for a mediator is the PI3K/Akt pathway that we also investigated in our study.

PI3K/Akt signaling is initiated when endothelial cells are subjected to shear stress. The shear stress-induced activation of this signal transduction pathway is dependent on VEGFR-2.[187] It is already known that PI3K/Akt signaling is responsible for both the shear stress-induced production of nitric oxide and the resistance to apoptosis of sheared endothelial cells.[38, 54] So far, there has been only one study to investigate the effect of this pathway on cytoskeletal dynamics in response to shear stress. In this study it was found that in non-confluent endothelial cells that migrate along with the direction of shear stress, PI3K signaling does not have an impact on actin dynamics.[208] However, the migratory response to shear stress in single cells is different from the cytoskeletal remodeling response of confluent endothelial cells to shear stress, especially in terms of upstream signal transduction.[187] It seems likely that PI3K is needed for shear stress-induced cytoskeletal remodeling in confluent cells, because it is downstream of VEGFR-2 activation.[187] Clear indications that PI3K is needed for mediating the effects of VEGFR-2 activation on the actin network come from studies in which VEGFR-2 was activated by $VEGF_{165}$ instead of shear stress. Treatment of endothelial cells with $VEGF_{165}$ induces formation of actin stress fibers, similar to what is seen in response to shear stress.[125, 166, 196] As we have also shown in this study, the formation of stress fibers after $VEGF_{165}$ treatment is mediated by Akt signaling.[125] What lies downstream of Akt signaling is not clear. Some groups report that the $VEGF_{165}$-induced stress fiber formation depends on the small GTPase Rac,[166] while others show that the Rho/p160ROCK pathway is responsible.[196] Despite these clear indications that the PI3K/Akt pathway is involved in the shear stress-induced formation of actin stress fibers, our study showed that inhibition of this pathway does not affect the actin rearrangements in endothelial cells that are subjected to shear stress. So there must be another pathway that tranduces the signal from VEGFR-2 to the small GTPase effector proteins. The activation of small GTPases in response to shear stress is known to be mediated by activation of integrins.[185] These transmembrane proteins are important for binding of the cell to the underlying matrix. This suggests that there exists an indirect signal transduction pathway, in which VEGFR-2 first activates integrins, which in turn find new binding spots on the underlying matrix. Subsequently, this binding and clustering of integrins could facilitate the interaction of small GTPases with the membrane, which is their site of effect.[37]

In summary, using our microfluidic assay, we have contributed to the understanding of the intracellular pathways that play a role in the cytoskeletal remodeling in response to shear stress. The results in our study confirm findings by others and add to

the existing knowledge by showing that the PI3K/Akt pathway is not necessary for inducing cytoskeletal remodeling in response to shear stress.

Despite all the recent advances in microfluidic technology, application of microfluidic technology for answering biologically relevant questions has been limited.[134] This is probably due to both the effort needed to build and calibrate the more complicated microfluidic set-ups and the potential differences in cell physiology when working at a microfluidic scale instead of with conventional methods. The former problem does not really apply to our study, because we use such a simple microfluidic design that it is just as easy to set up as the conventional large-scale flow chamber. The second concern about differences in cell physiology can in principle be addressed using two alternative approaches. Firstly, by investigating important, comprehensive cell physiological parameters, such as gene expression state and proliferation rate, in the microfluidic setting. After such a thorough characterization of cell physiology, the results of new experiments with this specific set-up are very likely to be reliable.[118] The second approach is to confirm previously reported results from macroscopic studies on a microfluidic scale. As long as these control measurements deal with the same area of cell physiology as the envisioned new experiments, the conclusions drawn from these new experiments are also likely to be valid. In our study, we used the latter of the two approaches. We confirm previously reported cell behavior and use this as verification of the applied method. This gives us the confidence to also trust the novel results that were generated with the same method.

Conclusion

To our knowledge, this study is the first in which microfluidic technology is applied for generating new biological insight in the endothelial response to shear stress. By treating endothelial cells with specific inhibitors during application of shear stress, it was found that the PKB/Akt pathway is not involved in the shear stress-induced cytoskeletal remodeling in these cells, even though it is involved in cytoskeletal remodeling in response to growth factors.

Microfluidic technology provides us with invaluable tools that have the potential to greatly increase the rate at which new discoveries in cell biology are made. We hope that our approach in this study sets an example for other biologists to also apply microfluidic technology for generating biological knowledge in the future.

Chapter 5: VEGFR-2-dependent, transient micromechanical stiffening of endothelial cells in response to fluid shear stress

Manuscript submitted: A.D. van der Meer, Y. Li, M.H.G. Duits, A.A. Poot, J. Feijen, I. Vermes

Vascular endothelial cells form the inner lining of all blood vessels and play a central role in vessel physiology and disease. Endothelial cells are highly responsive to the mechanical stimulus of fluid shear stress that is exerted by blood flowing over their surface. In this study, the immediate micromechanical response of endothelial cells to physiological shear stress was characterized by tracking of ballistically injected, sub-micron, fluorescent particles. It was found that the mean squared displacement (MSD) of the particles decreases by a factor 1.5 within 10 minutes after the onset of shear stress. This decrease in particle motion is transient, since the MSD returns to control values within 15 to 30 minutes after the onset of shear. The immediate micromechanical stiffening is dependent on activation of the vascular endothelial growth factor receptor (VEGFR)-2, because inhibition of the receptor abrogates the micromechanical response. It is known that the cytoskeleton is necessary for inducing the acute, functional response to shear stress in endothelial cells. Therefore, further characterization of the cytoskeletal changes in response to shear stress is important for gaining understanding of endothelial physiology.

Introduction

Vascular endothelial cells form the inner lining of all blood vessels. Under physiological conditions, endothelial cells are constantly subjected to fluid shear stress, which is generated by blood flowing over their surface. This shear stress stimulus has an effect on virtually every important aspect of endothelial biology.[107] Proliferation,[100] apoptosis,[38] migration,[104] differentiation,[211] permeability,[80] inflammation,[170] they are all affected by the application of shear stress. Not surprisingly, shear stress is an important factor in the development of vascular disease.[8, 93, 117] Especially the nature of the shear stress stimulus has been found to be important in vascular pathophysiology: high shear stress leads to properly functioning endothelial cells, while low and fluctuating shear stress without a clear net direction promotes endothelial dysfunction.[107] As a result, major vascular pathologies like atherosclerosis are predominantly localized to regions of the vascular tree with disturbed blood flow patterns.[93]

Studying how changes in shear stress are sensed by the endothelial cell, how the signal is transduced into the cell and how this eventually induces physiological changes, will lead to increased understanding of vascular disease and may provide new methods and targets for treatment or prevention. Because steep oscillations in shear stress magnitude and direction seem to be essential for inducing endothelial dysfunction, it is particularly interesting to characterize the response of endothelial cells to shear stress on time scales ranging from seconds to minutes. These timescales correspond with in vivo variations in shear stress magnitude due to the cardiac cycle and due to exercise-induced changes in cardiac output, respectively.

One of the aspects of the response of endothelial cells to shear stress is a change in cytoskeletal structure.[120] The effects of long term application of shear stress on the endothelial cell cytoskeleton and general cell morphology are well-characterized. Within six to twenty-four hours, endothelial cells elongate and orient in the direction of the flow. Thick bundles of actin fibers that are aligned with the shear stress direction appear in the cell body and the microtubule organizing center positions itself on the upstream side of the cell. These structural cytoskeletal changes not only lead to an altered endothelial morphology, but also induce an increase in cell stiffness, as shown by micropipette aspiration and atomic force microscopy.[63, 154, 155] However, the short term effects of shear stress on the cytoskeleton and the mechanical properties of endothelial cells are less well-studied. This is mainly due to the lack of tools to simultaneously apply shear stress and probe changes in cytoskeletal dynamics. The main technique for real-time tracking of cytoskeletal adaptations is introduction of fluorescently labeled cytoskeletal proteins into the cell. The dynamics of these labeled cytoskeletal components can then be monitored with high-resolution time-lapse microscopy or advanced techniques like photo-activation of fluorescence and fluorescence recovery after photobleaching.[64, 126, 132] By using these techniques, it has been found that the cytoskeletal filaments displace in the shear stress direction within minutes after application of the stimulus.[64, 126] This shows that the shear stress stimulus is directly transmitted from the apical surface to the cytoskeletal network. Moreover, also within minutes after the onset of shear stress application, formation of lamellipodial protrusions has been observed.[126] The formation of these protrusions is an exemplification of shear stress-induced changes in cytoskeletal dynamics on very short timescales. On a slightly longer time scale, fifteen to thirty minutes after the onset of shear, actin turnover rates start to increase significantly, showing a prolonged cytoskeletal response to shear stress.[132] In summary, it is obvious that almost immediately after shear stress application, cytoskeletal dynamics are altered. Whether these short-term changes in cytoskeletal properties are also reflected in the mechanical state of the endothelial cell is more difficult to measure. The reason for this is that conventional techniques for measuring these properties, like micropipette aspi-

ration or atomic force microscopy, are impossible to combine with real-time shear stress experiments.

Recently, the technique of intracellular particle tracking has emerged as a real-time tool to probe the micromechanical dynamics of the cytoskeleton while applying various stimuli to cells.[207] The technique works by tracking the motion of endogenous particles (like lipid droplets or mitochondria) or injected sub-micron particles inside the cell cytoplasm. The sizes of the tracer particles are in the range of 0.2 to 0.5 μm, which is an order of magnitude larger than the apparent mesh size of the cytoskeleton, which is approximately 25 to 50 nanometer.[113, 114] Therefore, the particles do not diffuse freely inside the cell, but can be used to probe the viscoelastic properties of the intracellular environment. Changes in particle movement are caused by shifts in the balance between driving forces and the resistance offered by the cytoskeletal viscoelastic network. A decrease in average particle displacement is generally interpreted as an increase in stiffness of the particle microenvironment. Therefore, in this article, changes in particle dynamics will also be referred to as changes in "micromechanical stiffness". However, it should be noted that the direct link between particle motion and mechanical properties of the particle microenvironment is still under debate in the field. After all, particle motion within living cells may also be affected by active processes, especially when measuring the displacement of particles over a long interval (tens of seconds to minutes). The contribution of these active, non-thermal driving forces to particle dynamics is still under investigation.[61, 106, 124, 172] In addition, it should be stressed that a change in micromechanical stiffness can not simply be considered to reflect a change in the mechanical properties of the cell as a whole. A cell contains spatially heterogeneous structures like the nucleus, the actin cortex and the cell membrane, which do not affect the dynamics of most probing particles, but which do contribute to the mechanical properties of the cell. Intracellular particle tracking is a technique to specifically probe the micromechanical properties of the cytoskeleton, ignoring the contribution of cellular structures on a higher order.

To our knowledge, there have been two studies in which the technique of intracellular particle tracking was applied to monitor the response of cells to shear stress. By tracking endogenous particles in endothelial cells, Dangaria and Butler found an immediate increase of the amplitude of particle motion after the onset of shear stress.[29] On the other hand, when tracking ballistically injected particles, Lee, et al. found a significant decrease in the particle displacement when treating fibroblasts with shear stress for forty minutes.[98] The discrepancy between these two studies is most likely caused by the differences between the probe particles. There is compelling evidence that injected particles are embedded in the actin network, while endogenous particles are located in the direct vicinity of microtubules.[106, 198] Therefore,

changes in the motion of these two types of particles probably reflect changes in the dynamics of different components of the cytoskeleton. However, the differing results could also be caused by differences in cell type or the duration of shear stress application. Obviously, more research is needed to thoroughly characterize the micromechanical response of cells to shear stress and explain these opposing trends.

From the studies with fluorescently labeled proteins and the studies with intracellular particles, it is quite clear that cytoskeletal dynamics are directly affected when cells are subjected to shear stress for seconds or minutes. However, what signaling events precede these changes in cytoskeletal dynamics is still partly unknown. There are numerous studies that point to a role of small guanosine triphosphatases (GTPases) like Rho, Rac and Cdc42 as effector molecules in shear stress-induced cytoskeletal remodeling.[184] The activation of these effector pathways relies on the formation of new interactions between integrins and the substrate to which the cells are attached. Presumably, this binding to the basal substrate is facilitated by shear stress-induced intracellular activation of integrins.[163] This shear stress-dependent, inside-out activation of integrins has been shown to rely on the activation of a signaling complex, which is comprised of vascular endothelial (VE)-cadherin, platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial growth factor receptor (VEGFR)-2.[187] However, there have been no studies that directly link activation of this signaling complex to the immediate cytoskeletal response of endothelial cells to shear stress.

The goal of the study described in this paper was to investigate the immediate changes in cytoskeletal dynamics in endothelial cells in response to shear stress, using the relatively new tool of particle tracking. In addition, it was investigated what upstream signaling pathways are involved in mediating shear stress-induced changes in cytoskeletal properties. The main conclusion of the study is that the motion of ballistically injected particles in endothelial cells decreases almost immediately when these cells are subjected to shear stress. However, this is a transient effect. Moreover, it is shown that these effects on particle dynamics are dependent on activation of VEGFR-2. The study identifies VEGFR-2 as an essential component in the short-term cytoskeletal response of endothelial cells to shear stress. This receptor and its downstream signaling pathways are therefore interesting candidates for further studies into shear stress-induced pathophysiology.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs, Tebu-bio, The Netherlands) were maintained in culture in Endothelial Growth Medium-2 (EGM-2, Endothelial

Basal Medium with 2% fetal bovine serum and a number of additives, Lonza Benelux, The Netherlands). All cell culture surfaces in this study were coated with 2 mg/ml partially purified fibronectin solution (Sanquin, Amsterdam, The Netherlands) for 30 minutes to two hours before use. Cells were kept in a humidified incubator at 37°C and 5% CO_2 and used in experiments when they were between passage 2 and 6.

Microfluidic channels

Microfluidic channels were prepared by pouring a Sylgard silicone elastomer (base:curing 10:1, Dow Corning, USA) on a silicon mold with micrometer-sized structures of epoxy-based SU-8 photoresist (60 μm × 500 μm × 1 cm, h × w × l). The silicone elastomer was crosslinked at 60°C for 16 hours and then peeled off from the mold. Holes were punctured in the silicone to reach the channel. Then, a closed channel structure was formed by treating both the silicone channel and a glass delta T culture dish (Bioptechs, Butler, PA, USA) with 100 W oxygen plasma for 60 seconds and pressing them together.

Ballistical injection of particles

Before particle injection, cells were allowed to form a confluent monolayer in 35 mm Petri dishes by overnight culturing in an incubator. The following day, monodisperse, red-fluorescent carboxylated poly(styrene) latex particles with 0.2 μm diameter (Invitrogen, Breda, The Netherlands) were ballistically injected into the cells. Ballistic injection was carried out following a protocol adapted from Panorchan, et al.[135] First, the particles were suspended at 2% (w/v) in ethanol. Then, the particles were spincoated onto microcarrier discs and the discs were left to dry overnight at room temperature. Next, both the Petri dishes with the cells and the microcarrier discs were placed in a Biolistic PDS-1000/He particle delivery system (Bio-rad, USA). When using optimized parameters for ballistic injection (vacuum level 28 mmHg, helium pressure 1350 psi, microcarrier disc-to-sample distance 3 cm), approximately 50% of the cells survived the injection procedure. After injection, the Petri dishes were flushed thoroughly three times to remove all non-injected particles. The cells were left in the incubator for 30 minutes before they were trypsinized and seeded in the microfluidic channels.

Culturing inside channels

A highly concentrated cell suspension (20×10^6 cells/ml) was pipetted into the channel. The microfluidic devices were overlaid with thick drops of EGM-2 to limit evaporation and placed in the humidified incubator for overnight cell attachment. The next day, cells were used in experiments.

Staining for VE-cadherin and F-actin

The cells inside the channel were fixed with 4% paraformaldehyde for 15 minutes. When staining for VE-cadherin, the cells were incubated with mouse anti-VE-cadherin monoclonal antibodies (Santa Cruz Biotechnology, USA) in phosphate-buffered salt solution with 1 mg/ml bovine serum albumin and 1% Triton X-100 (Sigma, Germany) for one hour. After rinsing, the cells were incubated for one hour with Alexa488 goat anti-mouse antibody solution (Invitrogen, USA). When staining for filamentous actin, the fixed cells were incubated with 1 μg/ml phalloidin-FITC for 30 minutes. The resulting fluorescent staining was visualized with a Zeiss LSM 510 confocal laser scanning microscope.

Application of shear stress

Before applying shear stress, the whole set-up was assembled as follows. The delta T dish with the microfluidic device containing the cells was mounted on the heating stage (set at 37°C) of an UltraView LCI10 system (Perkin Elmer), in which a Yokogawa spinning-disc confocal unit is combined with a Nikon Eclipse TE-300 microscope. The microfluidic device was connected to a syringe filled with CO_2-independent medium (Invitrogen, USA) with 10% fetal bovine serum (Lonza Benelux, The Netherlands), 1× penicillin/streptomycin (Lonza Benelux, The Netherlands) and, in some cases, 5 μg/ml VEGFR-2 Kinase Inhibitor (CalBioChem, USA) by blunt needle connections (EFD, Inc., USA) and tygon tubing (Rubber BV, The Netherlands). The assembled set-up was left alone for one hour to bring the cells to static resting conditions. Then, the medium was pumped through the channel at 1.5 ml per hour. Shear stress on the cells was estimated to be 1 Pa by using the equation for shear stress in rectangular channels: $\tau = 6Q\mu/wh^2$, with τ shear stress in Pa, Q flow rate in m^3/s, μ viscosity of the medium (set at the viscosity of water at 37°C, $7 \cdot 10^{-4}$ Pa·s) and w and h, width and height of the channel in m, respectively. Particle tracking was performed both before and during the application of shear stress.

Multiple particle tracking

15 to 40 probe particles per cell were visualized with a 100× (numerical aperture 1.3) oil immersion objective by exciting them with a 564 nm krypton laser and recording the fluorescent images with a 12-bit CCD camera (Hamamatsu Photonics, Germany) at approximately 17 frames per second. Typically, a movie of 60 seconds, or 1000 images was recorded. The spatial resolution of the images was 0.13 μm per pixel. The movies were analyzed using the publicly available particle tracking code[27] based on code by Crocker and Grier.[26] The change in position of particles between two different frames with a known time interval (the 'lag time') was tracked. By averaging over the squared displacements of all particles in the analyzed frames, the

mean squared displacement (MSD) could be obtained. MSDs were determined for the shortest possible lag time (1 / 17 fps = 0.06 s) and for longer lag times, up to 3 s. Sometimes aggregates of particles were observed; these were excluded from analysis. When analyzing the MSD of probe particles glued to a coverslip, the localization error of the detection technique was found to be approximately 15 nm (see also figure 16c).

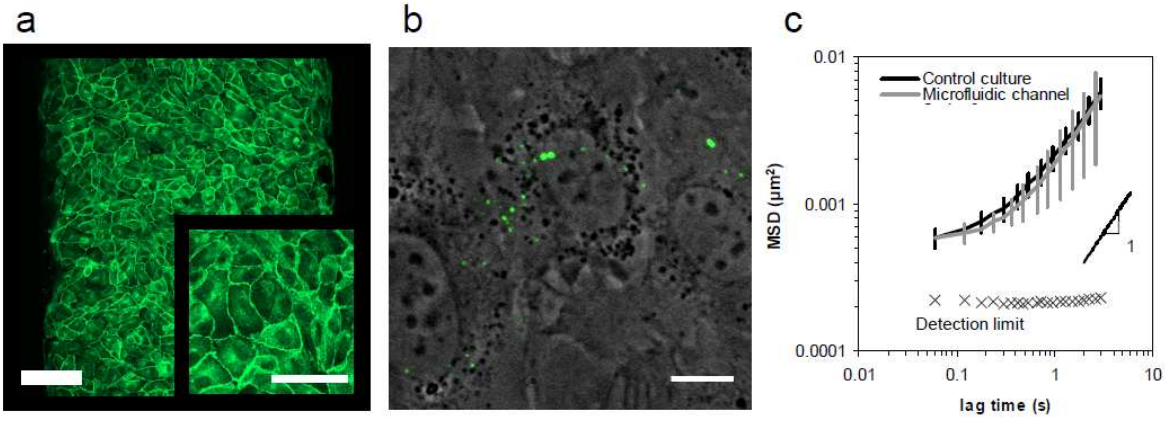

Figure 16: Characterization of endothelial cells in a microfluidic channel. a. Fluorescent micrograph of endothelial cells cultured in a 500 μm wide microfluidic channel. The cells were fluorescently stained for VE-cadherin. The staining pattern shows that the entire surface of the channel is covered with a monolayer of endothelial cells. Moreover, the cells have formed tight cell-cell contacts (inset). Scale bar, 100 μm. Scale bar inset, 50 μm. b. High magnification phase contrast image of endothelial cells in a microfluidic channel. The picture is overlaid with a fluorescent micrograph, which reveals the location of the ballistically injected fluorescent particles. Scale bar, 10 μm. c. Plot of MSD versus lag time. The curves show that the particle dynamics is the same, regardless of whether cells are cultured on normal cell culture dishes or in microfluidic channels. The curves exhibit a semi-plateau at short lag times and show near-linear behavior at longer lag times. A line with a slope of 1 was plotted for reference of purely viscous behavior. The observed MSD is well above the detection limit of the system, as determined by tracking particles that were glued to a coverslip.

Results

HUVECs with ballistically injected fluorescent particles were cultured overnight in 500 μm-wide microfluidic channels before performing shear stress experiments. In order to confirm that overnight culturing of HUVECs in these channels yields a confluent monolayer, a staining for VE-cadherin was performed (figure 16a). The staining pattern showed that cells covered the entire cell culture surface and exhibited tight cell-cell contacts (figure 16a, inset). The typical morphology of the cells as observed with high-magnification, phase-contrast microscopy is shown in figure 16b. This figure also reveals the pattern of fluorescently labeled particles inside the cell cytoplasm. Moreover, we checked whether particle dynamics in cells that are cultured inside a microfluidic channel are similar to the previously determined[106] dynamics of

cells on macroscopic surfaces (figure 16c). We found overlapping MSD lag time curves, showing that particle dynamics are the same, regardless of cell culture environment. In the MSD lag time curves, two characteristic regions can be observed. For short lag times (0.06 – 0.2 s), a plateau region for particle MSD is found. This is characteristic of elastic properties in the particle microenvironment. For longer lag times (0.2 – 3 s), an almost linear relation between lag time and MSD is observed. This is indicative of a more viscous particle microenvironment. This type of lag time-dependent behavior of particle MSD is characteristic for particles that are embedded in the cytoplasm of living cells, and has been associated with the apparent viscoelasticity of the cytoskeleton.[207]

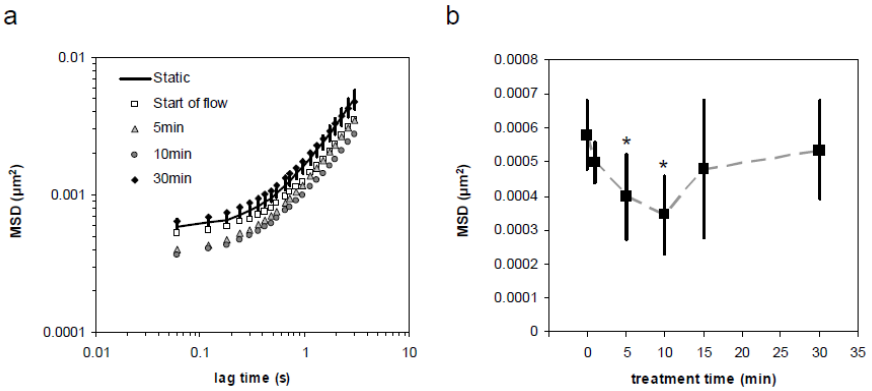

Figure 17: The effect of shear stress on particle MSD in endothelial cells. a. Typical example of MSD curves at different timepoints before and after application of a fluid shear stress of 1 Pa. The overall shape of the curves does not change significantly, but the MSD amplitude decreases significantly after 10 minutes. After 30 minutes of treatment, the MSD values have returned to control levels. b. Plot of the shortest lag time MSD (0.06 s) versus the duration of shear stress treatment. Time point zero indicates the MSD under static conditions. All data points are averages of four independent experiments. Error bars denote standard deviations. Asterisks denote data points that differ significantly from the static control (Student's t-test, $p < 0.05$).

Using the microfluidic cell culture system described in the previous paragraph, the effect of shear stress on cultured endothelial cells was studied. Before applying shear stress, particle tracking was performed to obtain control values for particle MSD under static conditions. The rest of the particle tracking was performed while applying shear stress. When the endothelial cells were subjected to a physiologically relevant shear stress of 1 Pa, an immediate and transient decrease in particle MSD was observed over the time course of 30 minutes (figure 17a). For further analysis, the MSD of the shortest lag time (0.06 s) was deemed to be the most reliable indicator for changes in the mechanical microenvironment of the particles. At these short lag times, the chances of including effects of possible active driving forces are relatively small.[124] The short lag time MSD of four different experiments was averaged and plotted versus duration of shear stress application (figure 17b). When analyzing this graph, it becomes clear that the shear stress-induced decrease of MSD was statistically significant after 5 minutes

of shear stress application. After 10 minutes, the MSD reached a minimum value, which is more than one and a half times lower than the particle MSD before application of shear stress. Within 15 to 30 minutes after the onset of shear, the particle MSD returned to control values.

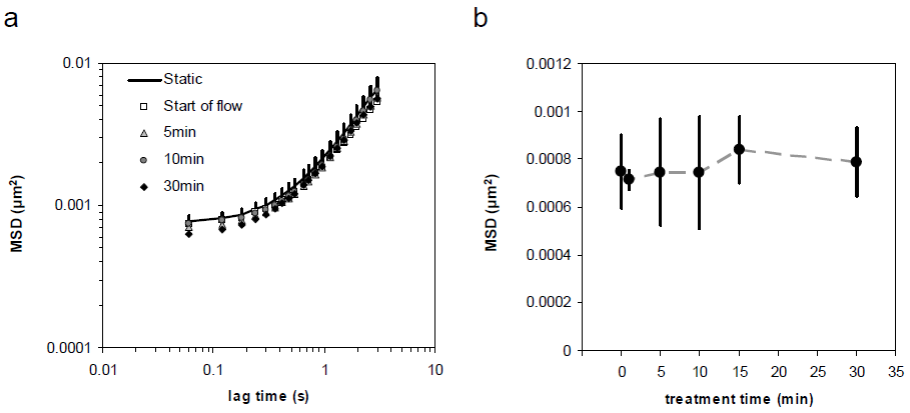

Figure 18: The effect of VEGFR-2 inhibition on shear stress-induced changes in particle MSD. a. When treating cells with a shear stress of 1 Pa in the presence of an inhibitor of VEGFR-2, no significant changes in the shape of the MSD curves can be observed. b. The plot of the shortest lag time MSD versus shear stress duration confirms the insensitivity of VEGFR-2 inhibitor-treated cells to the shear stress signal. All data points are averages of three independent experiments. Error bars denote standard deviation.

In order to identify upstream signal transduction events that mediate the shear stress-induced transient decrease in particle MSD, the experiments were repeated in the presence of an inhibitor of VEGFR-2. After one hour of pre-incubation with 5 µg/ml VEGFR-2 Kinase Inhibitor, a shear stress experiment was performed. In unsheared cells, VEGFR-2 inhibition led to a small increase in MSD. In sheared cells, inhibition of VEGFR-2 completely abrogated the changes in particle MSD that were observed in control cells (figure 18). Apparently, the effects that shear stress has on the particle microenvironment do not take place when VEGFR-2 is inhibited.

Because the ballistically injected particles are mainly considered to be a probe for the dynamics of the actin cytoskeleton,[106] the morphology of the actin cytoskeleton before and after shear stress application was investigated. When cells were cultured under static conditions, an intense fluorescent staining of the actin cortex on the cell periphery was observed (figure 19, left). However, when cells were subjected to shear stress for 10 minutes, dense, thick bundles of actin microfilaments, called stress fibers, were formed in the cell body (figure 19, middle). These dramatic actin rearrangements were still present after 30 minutes of shear stress (figure 19, right). Moreover, when cells were subjected to shear stress in the presence of the inhibitor of VEGFR-2, the changes in actin staining patterns were similar to the control situation. It can be concluded that the differences in particle dynamics between various

timepoints and treatment conditions are not reflected in the overall staining patterns of the actin cytoskeleton.

Discussion

In this study, the dynamics of ballistically injected particles were used to characterize the effect of fluid shear stress on the cytoskeletal micromechanics of endothelial cells. It was found that treatment of endothelial cells with physiological levels of shear stress leads to a rapid and transient decrease of MSD of the injected particles, reflecting a cytoskeletal micromechanical stiffening. The most important finding of this study is that the stiffening depends on VEGFR-2 activation.

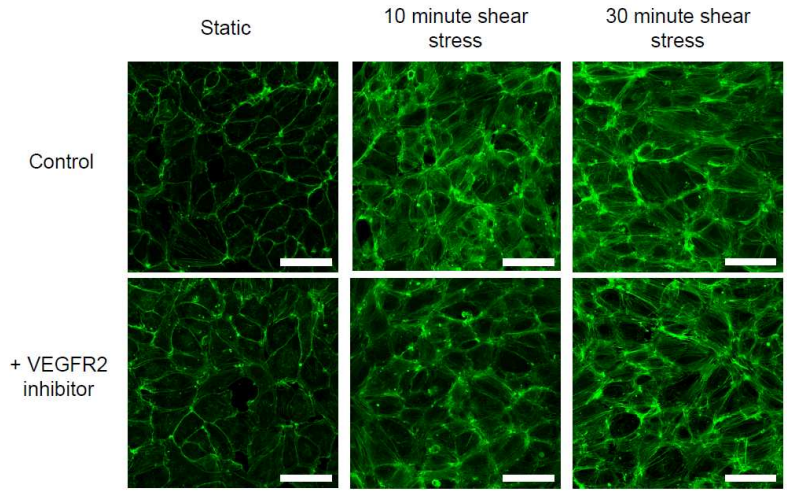

Figure 19: Shear stress-induced remodeling of the actin cytoskeleton in the absence and presence of an inhibitor of VEGFR-2. When cells are treated with a shear stress of 1 Pa for 10 minutes, thick actin stress fibers appear in the cell body and periphery. This actin staining pattern is still present after 30 minutes. Whether shear stress is applied in the absence (top) or presence (bottom) of an inhibitor for VEGFR-2 has no effect. Scale bars, 50 μm.

There have been two other reports in literature in which the method of particle tracking was used to probe the changes in cytoskeletal micromechanics in response to shear stress. The results in this study are most consistent with the results reported by Lee, et al., who reported a Rho GTPase-dependent decrease in the motion of ballistically injected particles in non-confluent fibroblasts, 40 minutes after the onset of shear stress.[98] In contrast, Dangaria and Butler reported a shear stress-induced increase in the motion of endogenous particles in endothelial cells, which was evident within one minute of shear stress application and which disappeared after four minutes.[29] The experimental system that was used in the current study is more similar to the former study in terms of probe particles, while the cell type used in our study more closely resembles the cells used in the latter report. Based on the similarities in outcome between the former study and our current study, it seems that the main factor that dictates the outcome of a study into cell micromechanics is the type of probe particle that is being tracked. There is more evidence for large differences in the behavior of endogenous granules and ballistically

injected particles when using them as micromechanical probes. Recently, it has been reported that the non-inert nature of endogenous granules renders them sensitive to fluctuations of microtubules, with which they interact.[198] Moreover, the endogenous granules are sensitive to non-thermally driven motion.[97] An indication for this is that endogenous granules typically exhibit a MSD that is almost an order of magnitude higher than that of injected microparticles.[106] The chance that the microparticles as used in the study by Lee, et al. and the current study are also subject to major non-thermally driven motion is rather small. They are not encapsulated by endogenous vesicles, because they are ballistically injected. Moreover, they have a surface chemistry that limits binding affinity for actin filaments.[121, 183, 190] Taken together, we feel that the conclusion that shear stress induces micromechanical stiffening in endothelial cells is justified.

The direct cause for the decrease in particle motion remains to be elucidated. Because the particles are lodged within the actin cytoskeletal network,[106, 113, 140] the observed micromechanical stiffening must be caused by changes of the dynamics of the actin cytoskeleton, like increased crosslinking or increased contraction of the microfilament network, or changes in the rate of polymerization and depolymerization of microfilaments. Moreover, we can conclude that large-scale remodeling of the actin cytoskeleton is not the cause for the observed decrease in particle motion. Our fluorescence microscopy studies showed that thick actin stress fibers form in the cell body in the same period as the micromechanical stiffening occurs (figure 19). However, this staining pattern is still present hours after starting to apply shear stress,[107] while the micromechanical changes disappear within half an hour after the onset of shear. Additionally, there was no effect on the actin staining pattern when treating with an inhibitor of VEGFR-2, while inhibition of this receptor did impact particle dynamics in our system. These results suggest that the observed formation of actin stress fibers is secondary to the micromechanical stiffening of the actin network and is not the direct cause. A similar finding was reported by Lee, et al., who observed that overall actin reorganization was not involved in mediating the micromechanical response of cells to shear stress. Instead, the actin contractile apparatus was found to be the main factor responsible for inducing the micromechanical stiffening.

A key observation in this study is that the shear stress-induced changes in particle dynamics were abrogated by incubating the cells with an inhibitor of VEGFR-2. VEGFR-2 is one of the first proteins to be activated in response to shear stress, because it is part of a mechanosensory signaling complex.[187] The activation of this signaling complex has been identified as an essential event for the induction of long-term cytoskeletal remodeling and of cell alignment with the direction of flow. Moreover, VEGFR-2 is essential for shear stress-mediated activation of protein kinase B (PKB/Akt) and endothelial nitric oxide synthase.[79] These molecules are important in

repressing endothelial cell apoptosis and inducing vasodilation. In short, VEGFR-2 is a central molecule in the endothelial response to shear stress and VEGFR-2-induced signaling has a broad impact on a lot of physiological aspects of this response. Now that we have also identified VEGFR-2 as an essential molecule for inducing cytoskeletal stiffening in response to shear stress, two major questions open up. Via which pathways does VEGFR-2 affect the cytoskeletal micromechanics? And what is the physiological relevance of the observed effect?

Related to the first question: major players that modify cell mechanics by altering actin network structure and contractile status are proteins from the family of small GTPases, like Rho, Rac and Cdc42.[15, 62] For example, the Rho/Rho Kinase (ROCK) pathway has been identified as an important player in shear stress-induced stiffening of fibroblasts by Lee, et al.[98] In endothelial cells, it has been shown that shear stress can activate and inactivate small GTPases on the same timescale as we observe changes in micromechanics.[185, 186] The exact interplay of small GTPases in mediating changes in micromechanics can only be elucidated by specific inhibition and activation of the different classes of GTPases. If we assume, for now, that GTPases are essential for inducing the transient micromechanical stiffening of the endothelial cell, then how are they linked to VEGFR-2? The most obvious pathway is via new integrin binding. The shear stress-mediated activation of integrins depends on VEGFR-2 signaling.[187] Moreover, the response of small GTPases to shear stress is completely dependent on the binding of activated integrins to new sites on the basal matrix beneath the endothelial cells.[184-186] Interestingly, the time course of integrin activation in response to shear stress is exactly the same as the micromechanical stiffening that we have observed in this study.[186, 187] So, to sum up, the presumed pathway from shear stress to micromechanical stiffening leads from activation of VEGFR-2 in the mechanosensory PECAM-1/VE-cadherin/VEGFR-2-complex, to inside-out activation of integrins, new binding of integrins to the underlying matrix and, finally, to activation of small GTPase effector pathways, which induce the stiffening.

Then, the second question: what is the functional relevance of the observed effect? Numerous studies have shown that all cytoskeletal components, including actin microfilaments, are necessary for mediating the acute endothelial effects to shear stress, like the release of vasodilatants.[76, 110, 173] However, although the role of the cytoskeleton in mechanotransduction is well-accepted, the exact underlying mechanisms remain unclear.[111] Our results show that the cytoskeleton undergoes immediate, but transient, micromechanical changes in response to shear stress. It will be interesting to characterize this response further, for example by interfering with the cytoskeletal structure and linking the results to functional effects, like the release of vasodilatants. Regulation of vasodilatants is an important factor in the prevention of

vascular dysfunction and its resultant pathologies, like atherosclerosis.[5] Studying the cytoskeletal pathways involved will increase our understanding of endothelial physiology and may lead to new targets for treatment of vascular disease in the future. The current study has firmly established intracellular particle tracking as a useful tool for performing these types of studies.

Chapter 6: A microfluidic wound healing assay for quantifying endothelial cell migration

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Endothelial migration is an important process in the formation of blood vessels and the repair of damaged tissue. To study this process in the laboratory, versatile and reliable migration assays are essential. The purpose of this study was to investigate if the microfluidic version of the conventional wound healing assay is a useful research tool for vascular science. Endothelial cells were seeded in a 500 μm wide microfluidic channel. After overnight incubation, cells had formed a viable and confluent monolayer. Then, a wound was generated in this monolayer by flushing the channel with three parallel fluid streams, of which the middle one contained the protease trypsin. By analyzing the closing of the wound over time, endothelial cell migration could be measured. Although the migration rate was two times lower in the microfluidic assay than in the conventional assay, an identical 1.5 times increase in migration rate was found in both assays when vascular endothelial growth factor ($VEGF_{165}$) was added. In the microfluidic wound healing assay, a stable gradient of $VEGF_{165}$ could be generated at the wound edge. This led to a 2 times increase in migration rate compared to the untreated control. Finally, when a shear stress of 1.3 Pa was applied to the wound, the migration rate increased 1.8 times. In conclusion, the microfluidic assay is a solid alternative for the conventional wound healing assay when measuring endothelial cell migration. Moreover, it offers unique advantages, such as gradient generation and application of shear stress.

Introduction

Migration of vascular endothelial cells plays an important role in vasculogenesis and angiogenesis.[18, 96, 157, 164] It is therefore also a critical process in the development of a number of diseases, such as cancer, rheumatoid arthritis and certain retinopathies.[19] Endothelial migration is affected by many intracellular pathways, as well as extracellular stimuli, such as growth factors and mechanical stress.[3, 148] In order to increase understanding of the process, chemical, physical or biological factors are tested for their ability to stimulate or inhibit endothelial cell migration. To this end, solid and reliable in vitro assays must be available.

In the laboratory, a number of assays are used for quantification of endothelial migration.[108] The two most widely used assays are the Boyden chamber assay and the

wound healing, or scratch, assay. The former assay works by seeding endothelial cells on one side of a permeable membrane that separates two culture compartments. Subsequently, the number of cells that migrate through the membrane to the other compartment is used as a measure of cell migration. The wound healing assay works by growing endothelial cells in a confluent monolayer and physically introducing a wound by scratching the layer. Cells move into the artificially generated space to close this wound. The rate at which this happens is used to quantify cell migration. Both methods have the advantage that they are easy to use and only require standard laboratory equipment. The main advantage of the Boyden chamber assay in comparison to the wound healing assay is that gradients of soluble factors can be generated by adding those factors to only one of the two compartments. On the other hand, the advantage of the wound healing assay is that it is easy to combine with microscopy, which makes it possible to track individual cells or perform staining after quantifying migration. Microscopy is not possible in the Boyden chamber assay, because the membrane is non-transparent.

The wound healing assay is usually carried out in standard wells-plates. Recently, however, a microfluidic version of the wound healing assay was reported in literature.[129] In this assay, the cells were cultured inside a microfluidic channel instead of a culture well. Because the closed channel structure does not allow for any physical scratching of the cell layer, the wound in the cell layer was generated by introducing three parallel fluid flows into the channel. Two of these flows contained normal medium, whereas one of them contained the protease trypsin. Cells that were exposed to the trypsin-containing flow detached from the surface, while cells that were exposed to normal medium remained attached. Afterwards, migration of cells into the artificially introduced wound could be followed and quantified just like in a regular wound healing assay.

In the aforementioned study, the researchers focused on proof of concept. A fibroblast cell line and growth factor stimulation were used to show the feasibility and usefulness of the microfluidic migration assay. Our aim for this study was to establish the reported microfluidic assay as a mature alternative for the conventional wound healing assay. By evaluating the viability of endothelial cells, monolayer integrity and cell migration rate, it will be shown that the microfluidic assay performs just as well as the conventional assay. Moreover, we wanted to highlight the advantages of using a microfluidic approach. By using the microfluidic assay, a stable gradient of growth factors can be applied to the wound. For the first time in literature, the effect of such a gradient on the wound healing rate will be described. Also, it will be shown that in the microfluidic assay, endothelial cells can be easily subjected to shear stress while studying wound healing. Our study firmly establishes that the microfluidic

wound healing assay is a useful research tool in vascular science and will serve as a starting point for more studies by the vascular research community.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs, tebu-bio, The Netherlands) were cultured in a humidified incubator at 37°C and 5% CO_2. The cells were overlaid with Endothelial Growth Medium-2 (EGM-2, Lonza Benelux, The Netherlands) in culture plates that were coated with 2 mg/ml partially purified fibronectin (Sanquin Amsterdam, The Netherlands). When the cells reached confluence, they were detached with trypsin solution (0.05% (w/v) porcine trypsin, 0.02% (w/v) EDTA in phosphate buffered saline (PBS), Lonza Benelux, The Netherlands) and replated in new flasks or used in experiments. The cells were used in experiments when they were between passage 2 and 8.

Microfluidic device fabrication

Microfluidic channels of poly(dimethylsiloxane) (PDMS) were prepared by pouring a 10:1 degassed mixture of base and curing agent of a Sylgard 184 elastomer kit (Dow Corning, USA) on top of a silicon wafer with micrometer-sized SU-8 structures. These structures had the inverse shape of a long channel with dimensions 60 μm × 500 μm × 2 cm (h × w × l) separated on one side into three smaller inlet channels. After crosslinking of the network for 16 hours at 60°C, the PDMS was peeled off of the mold, treated with oxygen plasma in a plasma sterilizer at 100 W for 1 minute and bonded to a glass coverslip by pressing the PDMS and glass together. Common theoretical analysis[199] of the fluid flow profile inside the rectangular channel showed that wall effects were minimal and would only affect the row of cells closest to the walls.

Cell seeding

Prior to cell seeding, the microfluidic channels were coated for two hours with fibronectin solution and then flushed with EGM-2. A $20 \cdot 10^6$ cells/ml cell suspension was prepared by harvesting cells from a culture flask with trypsin solution, pelleting the cells by centrifugation (300 × g, 5 minutes), removing the supernatant and resuspending in EGM-2. This cell suspension was pipetted into the channel, after which the microfluidic devices were overlaid with EGM-2 and incubated at 37°C and 5% CO_2. For the conventional wound healing assay, the endothelial cells were seeded in fibronectin-coated 24-wells-plates at a concentration of $40 \cdot 10^3$ cells/cm^2, overlaid with EGM-2 and placed in the incubator overnight.

Preparation of wounds

In order to prepare a wound, each of the three inlets of the microfluidic channel was connected to its own 5 ml syringe in a syringe pump using Tygon tubing. Two of the syringes contained Endothelial Basal Medium (Lonza Benelux, The Netherlands) with 2% fetal bovine serum (FBS, Lonza Benelux, The Netherlands) and the middle syringe contained trypsin solution (0.05% (w/v) porcine trypsin, 0.02% (w/v) EDTA in PBS, Lonza Benelux, The Netherlands). The contents of these syringes were pushed through the microfluidic channel at a total rate of 3 ml/hour for 15 minutes. After this, the pump was stopped, the channels were flushed with EBM-2, 2% FBS and placed in the incubator. When preparing wounds in the conventional wound healing assay, a single scratch was made in the endothelial monolayer using a micropipette tip. Subsequently, the cells were washed once with PBS and then incubated with EBM 2, 2% FBS at 37°C, 5% CO_2.

Quantifying cell migration

During the migration assay, the cells were taken out of the incubator every hour and placed under a Leica phase-contrast microscope with 10× objective. Pictures of fixed positions in the wounds were taken with a Canon digital camera that was mounted on the microscope. Subsequently, the wound area in each picture was determined by outlining the wound and measuring the area using NIH ImageJ image analysis software.[1] From the wound area, the average wound width could be obtained by dividing the area by the length of the analyzed region. The obtained wound widths were plotted against time in Microsoft Excel software and a linear fit was generated for each dataset. The slope of the linear fit was used as a measure of cell migration.

Affecting cell migration with growth factors and shear stress

To study the effect of growth factor stimulation on cell migration, medium containing 100 ng/ml $VEGF_{165}$ (Sigma, The Netherlands) was pipetted into the well or the microfluidic channel. Care was taken to introduce this medium into the channel at a low rate of several microliters per minute, so as not to disturb the freshly prepared wound. In some experiments, a gradient of $VEGF_{165}$ was generated in the microfluidic channel. This was accomplished by connecting three syringes to their respective inlets. The middle syringe contained EBM-2, 2% FBS with 100 ng/ml $VEGF_{165}$, whereas the other two syringes contained only EBM-2, 2% FBS. The total flow rate through the channel was adjusted to 0.3 ml/hour. The calculated shear stress (see below) on the endothelial cells caused by this flow is less than 0.2 Pa. Although a shear stress of this magnitude can be sensed by endothelial cells,[131] it is not high enough to affect chemotactic migration.[71]

When the effect of shear stress on endothelial migration was tested, EBM-2, 2% FBS was pushed through the channel at a rate of 2 ml/hour. A theoretical estimate for the shear stress on the endothelial cells can be given by using the calculation for wall shear stress in rectangular channels: $\tau = 6Q\mu/wh^2$, where τ is shear stress in Pa, Q is flow rate in m^3/s, μ is dynamic viscosity in Pa·s and w and h are the width and height of the channel in m. The medium viscosity can be approximated by the viscosity of water at 37°C and was set at $7 \cdot 10^{-4}$ Pa·s. This means that the shear stress that was applied to the endothelial monolayer was around 1.3 Pa, a value that lies within the range of physiological values.[144]

Fluorescence microscopy

All fluorescent microscopy studies were carried out using a Zeiss LSM 510 confocal laser scanning microscope. Cell viability staining was performed by incubating cells in EBM-2 with 2% FBS and a 1:1000 dilution of both components of a Live/Dead Kit (Invitrogen, USA) for 30 minutes. Viable cells were identified by green calcein staining, whereas the DNA of dead or damaged cells was labeled with red fluorescent ethidium homodimer-1.

Staining patterns for CD144 were obtained by fixating the cells for 15 minutes with HistoChoice fixating agent (Sigma, The Netherlands), incubating with primary anti-CD144 antibody (Santa Cruz Biotechnology, USA) in PBS with 1 mg/ml bovine serum albumin (Sigma, The Netherlands) and 0.1% Triton X-100 (Sigma, The Netherlands) for 30 minutes and finally incubating with Alexa488-labeled secondary antibodies (Invitrogen, USA) for another 30 minutes.

When staining for actin filaments, cells were fixated with 4% paraformaldehyde for 20 minutes. Subsequently, the cells were incubated with 1 μg/ml phalloidin-FITC in PBS with 1 mg/ml bovine serum albumin and 0.1% Triton X-100 for 30 minutes.

For estimation of the shape of a growth factor gradient, the microfluidic channels were connected to three 5 ml syringes: two containing PBS and one containing the same solution with 25 μg/ml 10 kDa dextran-rhodamine B (Invitrogen, USA). This dextran has a diffusion coefficient of approximately 85 $\mu m^2/s$.[7] This value lies in the same range as the diffusivity of $VEGF_{165}$ (133 $\mu m^2/s$)[116] and therefore serves as a reliable control for estimating the growth factor gradient shape.[10] The total flow rate through the channels was adjusted to 0.3 ml/hr, pictures were taken and the shape of the gradient was determined by plotting the profile with NIH ImageJ image analysis software.

Results

Seeding and cell viability

When seeding endothelial cells for a migration assay, it is important to cover the total culture area. If the cell density is too low, cell migration rate will be affected. As can be seen in figure 20a, when seeding endothelial cells in a microfluidic channel at a concentration of $20 \cdot 10^6$ cells/ml, they cover the entire surface after 24 hours. When working with microfluidic channels, it is important to realize that there is a very limited amount of medium present in the device (no more than 100 nanoliter in the channel itself, plus several microliters in the channel inlets). Therefore, diffusion of nutrients and waste products is limited when the channels are not constantly perfused with fresh medium. Still, in order to keep the protocol as simple as possible, cells were cultured under static conditions. As a result, it was necessary to study if cell viability is affected by long-term culturing without medium perfusion. In order to check cell viability, we used a calcein/ethidium homodimer Live/Dead assay. In this assay, living cells are labeled with green fluorescent calcein, whereas DNA of dead cells is labeled with red fluorescent ethidium homodimer. Using this assay, we determined cell viability after culturing the cells inside the microfluidic channel for several periods. As can be seen in figure 20a, after culturing of HUVECs in the device for 24 hours without refreshing the medium, the channel surface is still covered with viable cells. After 48 hours, the number of cells in the channel has dropped dramatically (figure 20b). The remaining cells are still viable, but have a stretched morphology. After 72 hours, there are still some viable cells in the channel, but it also contains a lot of fragments of dead cells (figure 20c). Based on these results, we decided to perform the microfluidic wound healing assay no more than 24 hours after seeding of the endothelial cells in the microfluidic device.

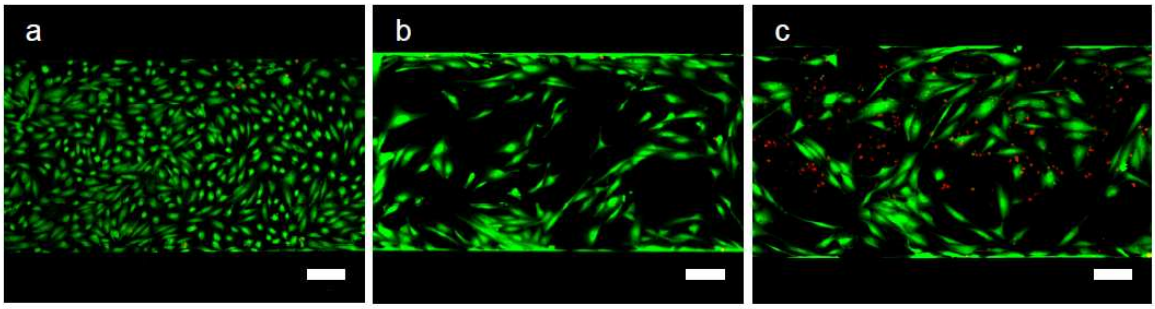

Figure 20: Cell viability assay on HUVECs in microfluidic channels. Green fluorescence indicates living cells, whereas red fluorescence indicates dead cells. When cells are cultured inside a microfluidic channel without refreshing medium for 24 hours (a), 48 hours (b) or 72 hours (c), viable cell confluency decreases over time. Scale bars, 100 μm.

Monolayer integrity

Another important parameter that needs to be controlled in a migration assay is the integrity of the endothelial monolayer.[108] Endothelial cells grow in tight monolayers with extensive cell-cell contacts. These cell-cell contacts are important in endothelial cell signaling and functioning.[36] It is therefore important that the endothelial cells have formed a confluent monolayer with tight cell-cell contacts before assessing migration in a wound healing assay. The integrity of the monolayer can be checked by staining for specific cell adhesion molecules, such as vascular endothelial cadherin (VE-cadherin, CD144). We performed fluorescent immunocytochemistry on HUVECs that were fixated at different time points after seeding to determine the cellular distribution of this adhesion molecule. As can be seen in figure 21, the staining of CD144 is localized exclusively at cell interfaces only after 18 hours. It is therefore important to incubate overnight after seeding to allow the endothelial cells the time to form a confluent monolayer.

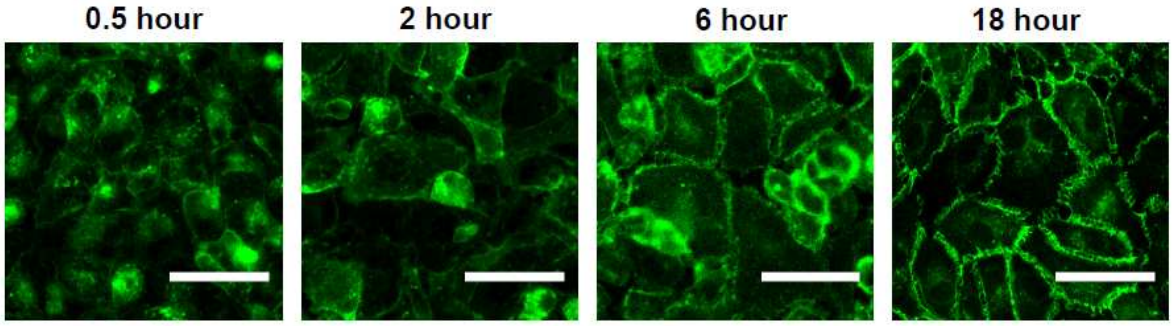

Figure 21: Fluorescent staining for vascular endothelial cadherin at different time points after seeding. Initially, the staining is found predominantly in the cell body, whereas the staining is found almost exclusively at cell contacts after longer time periods. Scale bars, 50 µm.

Wound preparation

Because the microfluidic channels are inaccessible for physical scratching, the wound was introduced by using three parallel fluid flows inside the channel. The stream in the middle contained the protease trypsin, while the streams on the sides contained medium with 2% serum. First, we checked if three parallel streams could be generated. As is shown in figure 22a, the channel could be flushed with three parallel streams with negligible mixing. This is a result of the very low Reynolds number (around 4) and short residence time of the medium (around 0.7 s) in the microfluidic channel. The only mixing between the laminar streams takes place by diffusion, but because the whole volume of the channel is replaced more than once per second, there is not enough time for visible mixing to occur. After applying these parallel streams with medium and trypsin to the endothelial monolayer, the middle of the channel was cleared from cells (figure 22b). In the conventional wound healing assay,

a wound was introduced in the monolayer of endothelial cells by scratching with the tip of a plastic micropipette (figure 22c). Both procedures result in similar wounds (figure 22b, c).

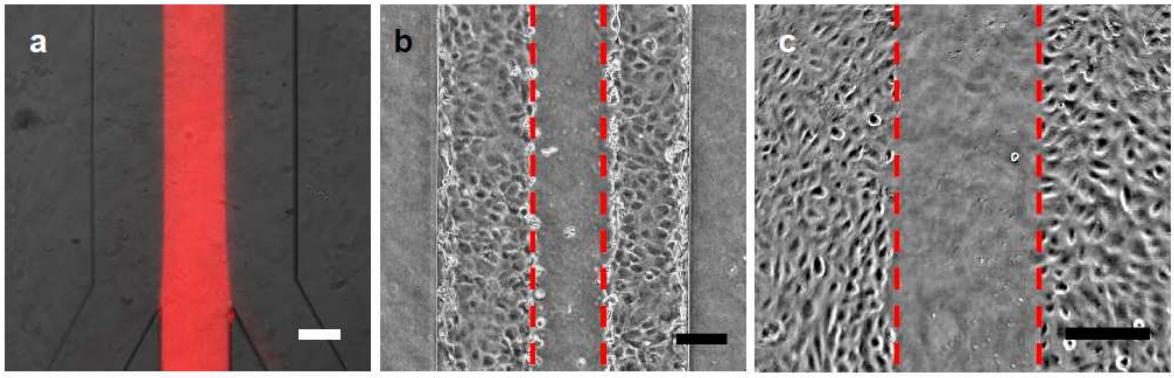

Figure 22: Inducing artificial wounds in endothelial cell monolayers. a, Fluorescent micrograph of a dextran-rhodamine B-containing fluid stream shows that no visible mixing of parallel fluid streams occurs in the microfluidic channel. Scale bar, 100 μm. b, Micrograph of a wound that was prepared by treating an endothelial monolayer with parallel trypsin- and serum-containing fluid flows in a microfluidic device. Scale bar, 100 μm. c, Micrograph of a wound that was prepared by scratching a monolayer in a wells-plate with a pipette tip. Scale bar, 250 μm.

Cell migration rate

Once a wound had been introduced in the endothelial monolayer, the migration rate could be quantified by taking pictures at fixed intervals. The wound size decreases over time and the rate at which this happens was taken as a measure of endothelial cell migration. Microfluidic channels are compatible with phase-contrast microscopy and it is therefore no problem to determine wound sizes (figure 23a). We found that the migration rate in microfluidic devices is stable over multiple hours, so this rate can be reliably quantified (figure 23b). We found that the average migration rate of HUVECs in the microfluidic wound healing assay is 12 μm/hour (figure 24). In the conventional wound healing assay in wells-plates, the average migration rate is 25 μm/hour.

Growth factor stimulation

One of the most important applications of migration assays is to assess the effects of drugs and other factors on the process of endothelial migration. In order for the microfluidic assay to be a viable alternative for the conventional assay, the measured effects should be similar. Therefore, we compared the effect of stimulation with $VEGF_{165}$ on endothelial migration in both assays. As summarized in figure 24, stimulation with $VEGF_{165}$ led to an increase in migration rate from 25 to 35 μm/hour in the conventional assay. In the microfluidic assay, these values were 12 μm/hour and 18 μm/hour, respectively. It is clear that the migration rate in the mi

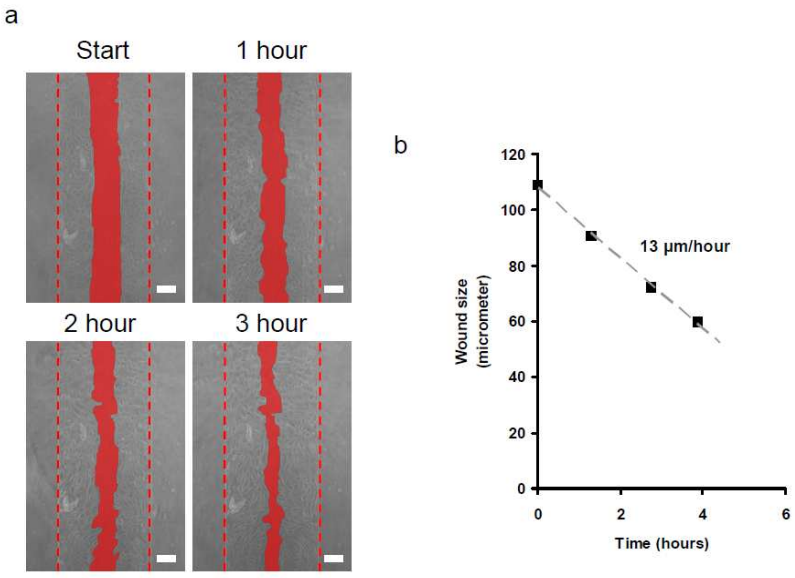

Figure 23: Typical example of wound size analysis. a, Micrographs of microfluidic channels at different time points after generating the wound. The wound area has been outlined and pseudo-colored in red. Scale bars, 100 µm. b, Plot of wound size versus time. The slope of this plot is a measure of endothelial migration rate.

crofluidic assay is lower than in the conventional assay. However, in both cases the addition of $VEGF_{165}$ led to a 1.5 times increase in cell migration rate.

A disadvantage of the conventional wound healing assay is that it is impossible to generate and study the effect of gradients of growth factors. Endothelial cells do respond to growth factor gradients in determining the direction of migration.[10] In the microfluidic version of the wound healing assay, it is possible to generate these gradients. When growth factors are added to the middle fluid stream and the flow rate is sufficiently low to allow for mixing between the streams to occur by diffusion, stable growth factor gradients are realized inside the channel. In order to assess the shape of such a gradient, a fluorescently labeled dextran with diffusivity similar to $VEGF_{165}$ was added to the middle stream. By checking the pattern of dextran with fluorescent microscopy and plotting the intensity of fluorescence, the shape of the gradient could be determined (figure 25). When a gradient with this shape was applied to a freshly prepared wound, the migration rate was significantly higher than when subjecting endothelial cells to stable concentrations of $VEGF_{165}$ (figure 24).

Shear stress application

In vivo, endothelial cells are constantly subjected to shear stress, which is caused by the blood flowing over their surface. When performing a conventional wound healing assay in wells-plates, it is difficult to assess the effect of this stimulus. To perform such an assay, spinning discs need to be fit onto the wells-plate.[72] Using this equipment, a non-uniform shear stress can be applied to the monolayer. However, in the microfluidic assay, it is enough to connect the channel to a syringe pump after

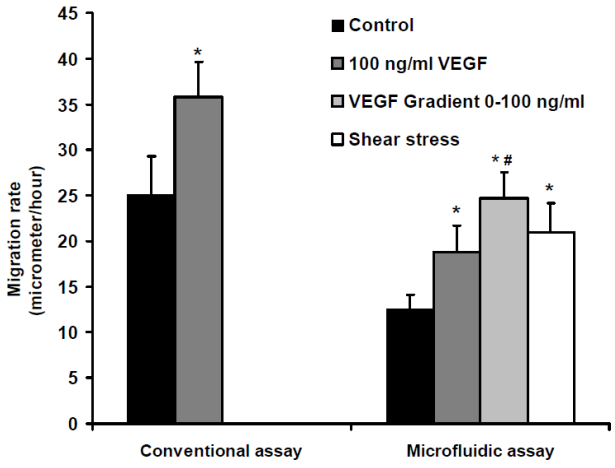

Figure 24: Comparison of migration rates in different assays under different conditions. In the conventional and microfluidic wound healing assay, migration was quantified under control conditions and in the presence of 100 ng/ml $VEGF_{165}$. In the microfluidic assay, migration was also quantified in the presence of a $VEGF_{165}$ gradient and while applying a shear stress of 1.3 Pa without a $VEGF_{165}$ gradient. All bars are averages of three independent experiments. * denotes a significant increase compared to control values (Student's t-test, $p < 0.05$), # denotes a significant increase compared to 100 ng/ml $VEGF_{165}$ treatment (Student's t-test, $p < 0.05$).

preparing the wound. In this way, physiologically relevant shear stresses can be generated using flow rates from 200 µl/hour to 2 ml/hour. As is shown in figure 24, applying a shear stress of 1.3 Pa leads to a significant increase in migration rate. Moreover, cell morphology during migration is different (figure 26). Staining of actin filaments shows that cells migrate into the wound along with the direction of the applied shear stress. Under static conditions, the pattern of actin filaments and the direction of migration are more random.

Discussion

Based on the results in this study, it can be concluded that the microfluidic wound healing assay is a good alternative for the conventional wound healing assay in wells-plates. Endothelial cells can be seeded in the microfluidic channel and form a tight monolayer of viable cells after overnight incubation. Wounds can be prepared by introducing three parallel fluid streams, one of which contains the protease trypsin. The rate of wound healing can be tracked over time and can be used to measure endothelial cell migration. We found the migration rate in the microfluidic assay to be about two times lower than in the conventional assay. This can be explained by the fact that the underlying substrates are completely different: in the conventional assay, cells migrate onto a tissue culture-treated polystyrene surface, while in the microfluidic assay the surface consists of trypsin-treated glass. In previous studies on endothelial cell migration, it has been found that both the average migration speed and the persistence of migration direction are higher on tissue culture-treated polystyrene than on glass.[74, 75, 90] Moreover, the results of an experiment with the conventional assay in which we compared wound healing rates on both tissue culture-treated polystyrene and glass coverslips, confirmed these differences (data not shown). It should be noted that these differences in migration rate are not a problem for the applicability of the microfluidic assay. Migration assays are used to study the effects of stimu-

lants or inhibitors on migration rate. So, as long as the effects of adding drugs or growth factors are the same in both assays, the microfluidic assay is still a good alternative for the conventional assay. We tested this by adding the growth factor $VEGF_{165}$ during wound healing in both assays. The stimulatory effect of $VEGF_{165}$ on endothelial migration is well-established and has been shown numerous times in literature when using a wound healing assay.[2, 65, 119] In our experiments, the effect of adding $VEGF_{165}$ was both qualitatively and quantitatively the same in both the conventional assay and the microfluidic assay. We found a one and a half times increase in migration rate when adding this growth factor, confirming both the known effect of $VEGF_{165}$ and validating the microfluidic assay as an alternative for the conventional assay.

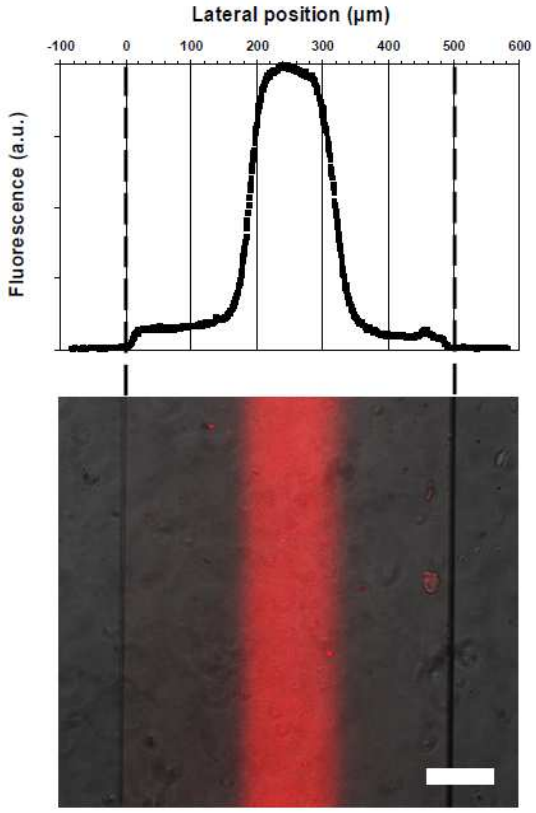

Figure 25: Shape of a stable gradient in the microfluidic channel. Bottom: Fluorescence micrograph of a microfluidic channel with rhodamine B-labeled, 10 kDa dextran in the middle stream and PBS in the other two streams. Scale bar, 100 µm. Top: Plot of relative concentrations versus lateral position in the channel. Relative concentrations were determined by analyzing the average fluorescence intensity of every lateral position in the fluorescent micrograph.

Gradients of $VEGF_{165}$ are important in the embryonic development of new blood vessels, as well as the formation of new blood vessels in adult tissue.[151] In order to generate more mechanistic insight into these processes on a cellular level, in vitro assays can be very useful. Using a Boyden chamber assay, endothelial migration in gradients of $VEGF_{165}$ can be quantified.[149] However, in this assay it is impossible to control the shape of the gradient or to visualize cells as they migrate into the gradient. Using microfluidics, it is possible to generate stable and tunable gradients of growth factors in devices that are compatible with microscopy.[102] Recently, this property of microfluidics was used to study chemotaxis of individual endothelial cells in gradients of $VEGF_{165}$.[10] The main finding of this study was that directed migration can be induced by applying gradients of $VEGF_{165}$ and that the shape of the gradient dictates the strength of the chemotactic response. The steepest gradient, starting at 0 ng/ml and increasing up to 50 ng/ml $VEGF_{165}$ over a distance of 400 µm was

found to induce the strongest migratory response. Our study is the first to assess the effect of a growth factor gradient on migration rate in a wound healing assay. The gradients that we applied increased from 0 to 100 ng/ml $VEGF_{165}$ over a distance of 150 µm. Because the gradients form at the interface of two parallel streams, they are located exactly on the edge of the induced wound. We found an increase of 30% in migration rate when applying these gradients instead of a fixed concentration of $VEGF_{165}$ of 100 ng/ml to the endothelial cells. This increase is probably due to an increased $VEGF_{165}$-mediated activation of migration-inducing signal transduction pathways on the side of the cell that is facing the wound.[125]

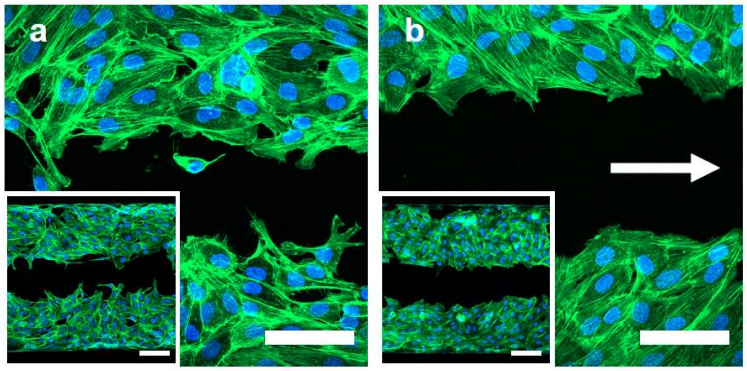

Figure 26: Actin filament staining of migrating endothelial cells under static and sheared conditions. a, Endothelial cells under static conditions migrate randomly into the wounded area. b, Cells that are being sheared orient their actin filaments along with the direction of the applied stress (arrow). Scale bars of close-ups, 50 µm. Scale bars of insets, 100 µm.

In vivo, endothelial cells are constantly subjected to shear stress that is caused by blood flowing over their surface. This shear stress is an important stimulus in endothelial cell functioning and has a big impact on blood vessel physiology.[107] There are a number of reports in literature on the effects of shear stress on endothelial migration rates in a wound healing assay.[3, 70] The principal finding of these studies was that shear stress enhances endothelial wound healing in vitro. This stimulating effect on endothelial migration is mediated by an enhanced activation of the Rho family of small GTPases.[70, 184] A secondary finding of these studies was that endothelial cells migrate along with the shear stress direction during wound healing. The increased wound healing rates are caused by this enhanced directional migration. Also in our study, we find that physiological levels of shear stress enhance wound healing. Moreover, morphological analysis of the migrating endothelial cells shows that they tend to orient their cytoskeletal components in the shear stress direction.

In conclusion, the microfluidic wound healing assay for endothelial cells that we describe in this study is a good alternative for the conventional wound healing assay. The microfluidic assay can be considered to be a highly useful laboratory tool for studying a lot of facets of endothelial migration. Moreover, the microfluidic version of the assay has some unique advantages compared to its conventional counterpart. Because of the small size, small amounts of cells and reagents are needed. This is es-

pecially useful when working with rare cell material (e.g. patient material) or expensive drugs. Furthermore, other cell-based applications of microfluidic technology are under active development.[44] This means that in the future, the microfluidic wound healing assay may be parallelized or combined with other microfluidic analysis tools in the same device: the so-called 'lab on a chip' concept.[205] Another advantage of the microfluidic wound healing assay is that it is compatible with high magnification microscopy. Moreover, stable and tunable gradients of growth factors and drugs can be generated and physiologically relevant shear stresses can easily be produced while studying migration. Given all these advantages, it will be interesting to see what other novel insights in vascular science will be produced using this new laboratory tool in the future.

Chapter 7: Microfluidic technology meets flow cytometry: the effect of shear stress on endothelial LDL uptake

Under review for publication: A.D. van der Meer, K. Vermeul, A.A. Poot, J. Feijen, I. Vermes

Acceptance of microfluidic technology in everyday laboratory practice by biologists is still low. One of the reasons for this is that the technology combines poorly with standard cell biological and biochemical analysis tools. Flow cytometry is an example of a conventional analytical tool that is considered to be incompatible with microfluidic technology and its inherent small sample sizes. In this study, it is shown that properly designed microfluidic devices contain cell populations that are large enough to be analyzed by flow cytometry. To illustrate this, the uptake of fluorescent human low density lipoprotein (LDL) by human endothelial cells that were cultured in a microfluidic channel was analyzed. It was found that the uptake of LDL by the cells increased linearly over time. Moreover, the uptake decreased when cells were pretreated with fluid shear stress inside the microfluidic devices. This study shows that microfluidic technology can be combined with conventional flow cytometry, while retaining the advantages of working with microfluidics such as low reagent use and dynamic cell culture conditions. This approach of combining microfluidic technology with conventional laboratory tools may contribute to greater acceptance of microfluidic devices in biological research.

Introduction

Microfluidic technology deals with the manipulation of fluid in channels with dimensions in the range of tens of micrometers. In the past two decades, microfluidic technology has been expanding rapidly into the field of cell biology.[44, 206] The technology offers an unprecedented control over cell culture micro-environment in terms of geometry, substrate and soluble factors, down to the single cell level. Moreover, the use of microfluidic technology significantly reduces the consumption of expensive reagents or rare primary cell material and can increase the throughput of experiments by parallel culturing in one device. However, so far, microfluidic technology has failed to generate a substantial impact on everyday practice in cell biological laboratories.[134] One of the proposed reasons for this lack of acceptance is that microfluidic technology is largely incompatible with broadly used cell biological assays. Sample sizes are so small that conventional analysis with Western or Northern blotting, gel electrophoresis, colorimetric assays and magnetic- or fluorescence-activated

cell sorting are difficult to achieve. One way of overcoming this difficulty is to develop microfluidic versions of these assays that are compatible with small sample sizes. For example, experimental microfluidic flow cytometers[73] and electrophoresis devices[210] have already been developed. However, integrating this technology into the cell-based microfluidic devices will increase the complexity of the systems and will thereby raise the threshold for use of this technology by biologists even further. It seems more promising to start by integrating microfluidic technology with conventional analysis tools, like plate readers and flow cytometers. Significant progress is being made by using this approach. For example, microfluidic devices that are compatible with conventional plate readers are under active development.[213] In the study described in this communication, we show that microfluidic devices harbor sample sizes that are large enough to be analyzed with conventional flow cytometry. As an example, we show that flow cytometry can be used to follow the uptake of human low density lipoprotein (LDL) by endothelial cells in microfluidic channels. We feel that integration of microfluidic technology with conventional analysis tools is an essential step in inducing widespread acceptance of microfluidic technology in the field of cell biology. The methods described in this study may bring this acceptance a step closer.

Methods and Results

Human umbilical vein endothelial cells (Tebu-Bio, The Netherlands) were cultured in Endothelial Growth Medium-2 (Lonza Benelux, The Netherlands) on surfaces that had been pre-coated with partially purified human fibronectin (Sanquin, Amsterdam, The Netherlands). When used in experiments, the cells were detached from the surface by trypsinization (using saline solution with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA) and spun down at 300 × g for 5 minutes. The cell pellet was resuspended in growth medium to reach a concentration of $20 \cdot 10^6$ cells/ml. This highly concentrated cell suspension was pipetted into a fibronectin-coated microfluidic channel (w × h × l, 500 μm × 60 μm × 20 mm). The microfluidic channels consisted of a slab of polydimethysiloxane (PDMS) bonded to a glass coverslip and were prepared by common soft lithographic techniques. In short, a 10:1 mixture of base and curing agent of a Sylgard 184 elastomer kit (Dow Corning, USA) was poured onto a silicon mold with micrometer-sized structures of epoxy-based SU-8 photoresist. The silicone elastomer was crosslinked for 16 hours at 60°C, peeled off of the mold and then bonded to a glass coverslip by treating both surfaces with oxygen plasma in a plasma sterilizer at 100 W for one minute and subsequently pressing them together. After the cell suspension was introduced into the microfluidic channel, cells were allowed to adhere to the glass surface for 6 hours. This yielded a channel surface that was completely covered with endothelial cells (figure 27a). Visu-

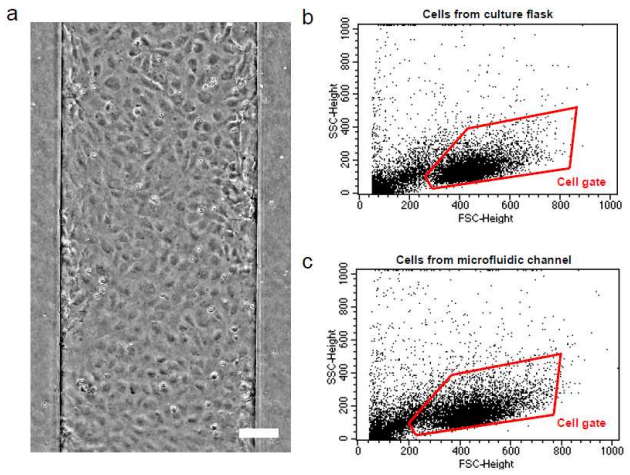

Figure 27: Endothelial cells in a microfluidic channel can be analyzed by flow cytometry. a. Phase contrast image of endothelial cells after 6 hours of culturing inside a microfluidic channel. Scale bar, 100 µm. b. Forward/sideward scatter plot of 10000 events in a sample from a cell culture flask. Based on size and granularity, the cells can be gated and separated from other particles in the plot. c. Same plot of a sample from a microfluidic channel. The scatter plot looks very similar to the plot of the sample from the cell culture flask.

alization by fluorescence confocal microscopy showed that cells were only attached to the bottom surface, not to the walls or upper surface (data not shown). With this information, a rough estimate for total cell number in the channel can be made. The diameter of an attached endothelial cell is approximately 40 µm. The surface area of a single cell is then given by $\pi \cdot 20^2 \approx 1250\ \mu m^2$ and the total amount of cells in the channel would be approximately $(500 \times 20 \cdot 10^3) / 1250 = 8000$ cells. In principle, if all cells could be recovered, this number should be high enough to perform conventional flow cytometric analysis. In order to test this, the cells in the channel were collected by pipetting trypsin solution into the microfluidic channel, letting the cells detach, flushing the channel three times with medium and centrifuging the resulting cell suspension. The centrifuged cell pellet was then resuspended in 100 µl phosphate buffered saline and analyzed using a conventional flow cytometer (BD FACSCalibur, BD Biosciences Europe, Belgium). In figure 27b and figure 27c, the forward/sideward scatter plots (indicating size and granularity of measured particles, respectively) of a sample from a normal cell culture flask and of a sample from a microfluidic channel are shown. The plots look almost identical. In both cases, the total amount of measured events was 10000, of which 6500 to 7000 were identified as cells by gating in the forward/sideward scatter plot. This shows that almost all cells could be recovered from the microfluidic channel for further analysis with flow cytometry.

Combining microfluidic devices and conventional flow cytometry has several advantages. Advantages of microfluidic technology are that only small amounts of cells and expensive reagents are needed to perform an experiment, that the channels are compatible with high magnification fluorescence microscopy and that cells can easily be subjected to fluid flow. The advantage of flow cytometry is that reliable fluorescent measurements can be performed with large sample sizes. This increases statistical robustness, which is usually a weak point of microfluidic technology with its in-

herent small sample sizes and predominantly microscopy-based methods for analysis. An experiment was devised to highlight these advantages and to gain insight into the uptake of LDL by endothelial cells.

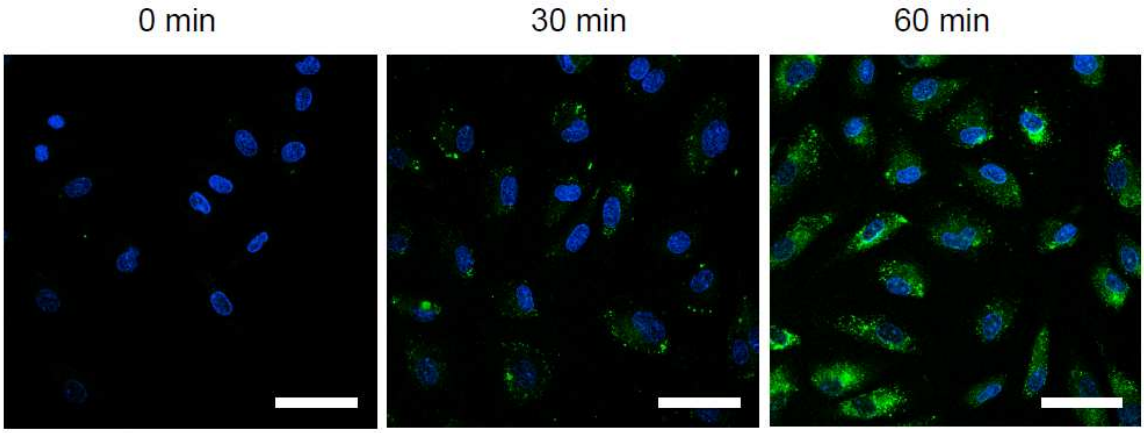

Figure 28: Fluorescent micrographs of endothelial cells that were exposed to BODIPY-LDL for different time periods. An increase in number and intensity of green fluorescent spots can be observed when cells were treated for longer time periods. Although visually appealing, the data is hard to quantify. Cell nuclei were counterstained with DAPI (blue fluorescence). Scale bars, 50 μm.

Again, cells were seeded in the microfluidic channels and allowed to adhere for 6 hours. Then, medium containing 10 μg/ml BODIPY-labeled human LDL (Invitrogen, USA) was added to the cells for several time periods. To analyze the staining pattern, cells were fixed for 15 minutes with 4% paraformaldehyde and the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Visualization by fluorescence microscopy on a Zeiss LSM510 confocal microscope showed that the endothelial cells did take up the fluorescently labeled LDL and that the uptake increased over time (figure 28). However, with this method, the degree of uptake was difficult to quantify reliably. Therefore, cells were incubated with LDL as described before, but were subsequently analyzed by flow cytometry. As shown in figure 29a, the increasing fluorescence of the entire cell population could be easily tracked over time. By plotting the percentage of fluorescently labeled cells (figure 29b), it was found that most cells became positively labeled between 5 and 15 minutes of treatment. However, the uptake of LDL continued at a constant rate for at least one hour, as shown by the linear increase in mean fluorescence of the population (figure 29c). This more in-depth characterization of the LDL uptake process exemplifies the accuracy that analysis by flow cytometry can provide.

Next, we were interested in testing the effect of fluid shear stress on endothelial LDL uptake. Fluid shear stress, caused by the flowing blood, has a profound impact on endothelial physiology.[107] Interestingly, atherosclerotic disease is predominantly

localized to regions of the vasculature with disturbed blood flow patterns.[60] The mechanism by which stable fluid shear stress protects against atherosclerosis, while disturbed flow is atherogenic, is still under active investigation. An effect of shear stress on endothelial LDL uptake may provide a direct link between shear stress and protection against atherosclerosis. After all, accumulation of LDL in the vessel wall is one of the first hallmarks in the development of the disease.[115] Active uptake and transcellular transport of LDL by endothelial cells is one of the processes that contribute to this accumulation, with paracellular transport[16] and increased retention of LDL molecules[159] as the other major contributors.

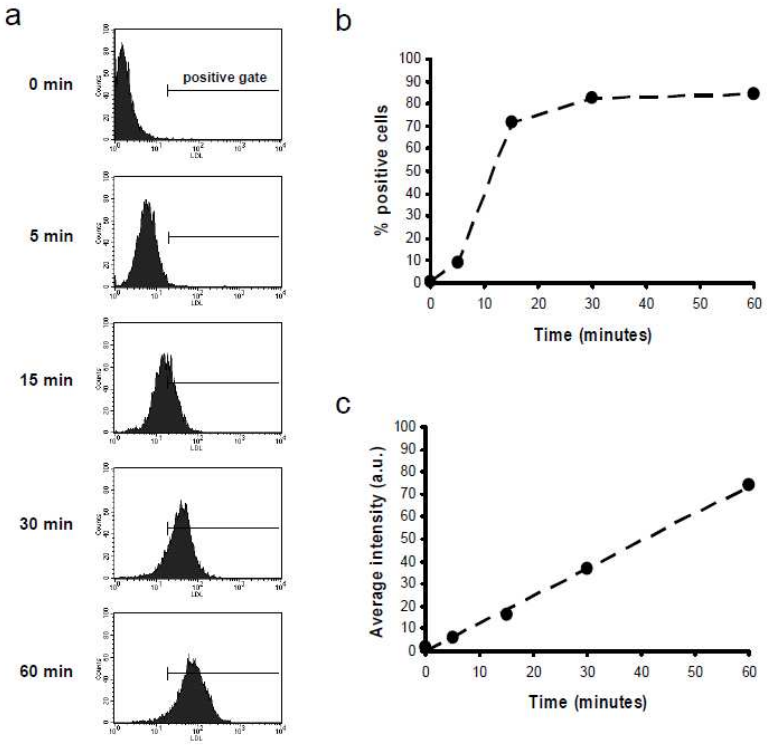

Figure 29: Flow cytometric analysis of BODIPY-LDL uptake by cells in microfluidic channels. a. Fluorescence frequency distribution plot of samples treated with LDL for different time periods. As treatment time increased (from top to bottom), the average fluorescence intensity (x-axis) of the cell population increased as well. A threshold value was set for defining which cells were positively labeled ("positive gate"). b. Plot of the percentage of positive cells in the samples from figure 3a. Most cells became positive between 5 and 15 minutes of treatment. c. Plot of the average intensity of the cell population for the samples from figure 3a. Over the time course of one hour, the average intensity increased linearly.

In order to apply shear stress, microfluidic channels were connected to a syringe pump, using blunt needle connections (EFD, Inc., USA) and tygon tubing (Rubber BV, The Netherlands). The shear stress on the endothelial monolayer was calculated by using the following equation: $\tau = 6Q\mu/wh^2$, with τ the shear stress in Pa, Q the flow rate in m^3/s, μ the viscosity of water at 37°C in Pa·s ($7 \cdot 10^{-4}$ Pa·s) and w and h the width and height of the channel cross section in m. When applying a flow rate of 1.5 ml/hour, the resultant shear stress was calculated to be approximately 1 Pa, a value that lies within the physiological range. This shear stress was applied to the endothelial cells inside the microfluidic channel for several time periods. After application of shear stress, the cells were treated for 10 minutes with 10 µg/ml fluorescently

labeled LDL. It was found that the uptake of LDL by sheared endothelial cells was lower compared to the uptake by cells that had been cultured in the channels under static conditions for the same time periods (figure 30). Moreover, the longer the shear stress stimulus was applied, the lower the uptake of LDL by the endothelial cells was (ANOVA, $p < 0.05$). To our knowledge, only one other study has been published in which the differences in LDL uptake between endothelial cells that had been cultured under static conditions and cells that had been pretreated with shear stress were investigated.[9] The qualitative results obtained by fluorescence microscopy in the study by Barkefors, et al. match our observations: pretreatment with shear stress lowers the uptake of LDL by endothelial cells in comparison to the statically cultured control. These observations definitely warrant further study to test the hypothesis that shear stress-induced differences in endothelial LDL uptake are involved in the onset of atherosclerosis.

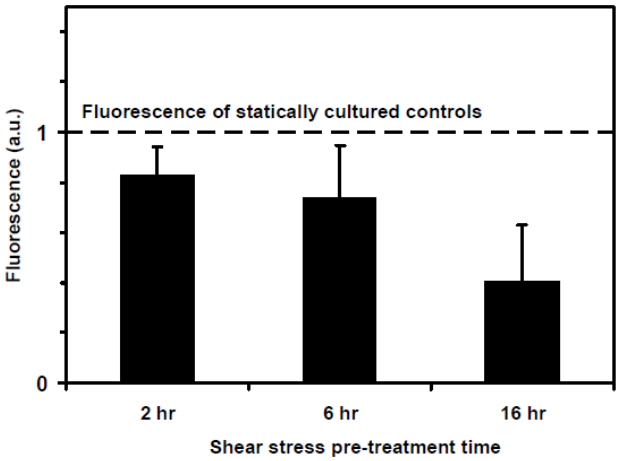

Figure 30: The effect of shear stress on endothelial LDL uptake. When cells were pre-treated with 1 Pa shear stress for different time periods inside microfluidic channels, subsequent uptake of LDL was lower than when cells were cultured under static conditions. This decrease in endothelial LDL uptake depends significantly on duration of shear stress pre-treatment (ANOVA, $p < 0.05$). For 2 hr pre-treatment, $n = 3$. For 6 hr and 16 hr pre-treatment, $n = 4$.

Conclusion

In conclusion, the results of this study show that combining microfluidic technology with conventional flow cytometry opens the door to cell biological experiments that take advantage of the strengths of both techniques. Showing the compatibility of microfluidic technology with conventional laboratory analysis tools lowers the threshold to apply the technology in cell biological research. We feel our study will contribute to more widespread use of microfluidic technology by cell biologists.

Chapter 8: Conclusions and Outlook

Authors: A.D. van der Meer, A.A. Poot, J. Feijen, I. Vermes

The results of the studies that are described in this thesis can be divided into two categories. On the one hand, there are purely cell biological results, which further our understanding of how endothelial cells respond to shear stress. On the other hand, there are results that demonstrate the applicability of microfluidic technology in studying endothelial cell biology.

In the response of endothelial cells to shear stress, one can discriminate three phases. First there is sensing, the process in which a purely mechanical signal is converted into a biological signal. Then there is transduction, or how the initial signal is converted into signals that can impact cell physiology. Finally, there is the response, which entails all functional changes that cells undergo when treated with the shear stress stimulus. In this thesis, most work focused on the phase of shear stress transduction. It was shown in chapter 3 that the scaffolding protein caveolin-1 is essential for early initiation of signal transduction in response to shear stress. Moreover, we found that its role probably lies in proper activation of vascular endothelial growth factor receptor (VEGFR)-2. The essential role of VEGFR-2 in shear stress-induced signal transduction was further highlighted in chapters 4 and 5, in which we described that both acute (chapter 5) and long-term (chapter 4) cytoskeletal remodeling in response to shear stress depends on activation of this protein. Moreover, the results in chapter 4 give a more detailed characterization of what signaling events lie downstream of the activation of VEGFR-2. It was shown that the effector protein p160Rho-associated Kinase (p160ROCK) is needed for shear stress-induced cytoskeletal remodeling, but that the remodeling process does not depend on the activation of a logical candidate for transducing the signal, phosphoinositide 3- kinase (PI3K). All these studies on the transduction of the shear stress stimulus help to increase our understanding of how shear stress induces its functional effects in endothelial cells. More understanding of shear stress-induced intracellular signaling facilitates the development of methods and drugs to modify the resulting functional response of cells to shear stress. In turn, by modifying the response, new methods of treating shear stress-related diseases, like atherosclerosis, may be obtained.

The other chapters in this thesis deal with the functional response of endothelial cells to shear stress. In chapter 6, we showed that the endothelial wound healing rate is increased by the application of shear stress. Furthermore, it was shown in chapter 7 that the endothelial uptake of low density lipoprotein (LDL) is lowered when the cells are pre-treated with shear stress. These results and other studies that focus on characterization of the endothelial response to shear stress may yield new links between the effect of shear stress on endothelial cells and the development of vascular

disease. For example, the accumulation of LDL in the vessel wall is a key event in atherosclerosis. Our finding that its uptake is lowered by treating with shear stress may help to develop insight into how shear stress affects atherogenesis.

Almost all studies that are described in this thesis were performed by using microfluidic technology. Some of the advantages of using this technology are obvious when reading the various chapters. For example, microfluidic technology can be easily combined with advanced, live-cell microscopy (chapter 5). Furthermore, the technology can be used to generate stable gradients of soluble factors (chapter 6). Moreover, with the rapid developments towards parallelization, automation and combination with analysis tools, using microfluidic technology in vascular cell biology will only become more beneficial. Interestingly, some of the biggest advantages of working with microfluidic devices are not explicitly mentioned in the results of the chapters, simply because they have no scientific value. Still, these advantages are worth mentioning and are important arguments for adopting microfluidic technology for biological research. By working with microfluidic devices, the time and cost required to perform an experiment are decreased dramatically. Fewer cells have to be cultured, lowering the necessary amount of medium and cultureware. Also, when cells need to be stained or analyzed, at least an order of magnitude less antibody or reagent is needed. Furthermore, when experimenting in microfluidic devices, so few cells are needed per condition that cell culture of primary cells is not the rate-limiting step anymore. Thus, a series of experiments can be performed in the same time that it would normally take to grow enough cells for performing just one experiment. Finally, assembling the microfluidic set-up is a lot simpler than the conventional flow chambers. This simple set-up is portable from incubator to microscope, even during an experiment. In short, microfluidic technology lowers cost, increases efficiency and simplifies the experimental set-ups.

The studies in this thesis have provided various examples of how to bridge the gap between microfluidic proof-of-concept studies and cell biological research. For example, the study with the microfluidic actin alignment assay described in chapter 4 is the first to employ microfluidic technology for gaining cell biological insight in the endothelial response to shear stress. Furthermore, in chapter 6, we report that the endothelial wound healing rate increases when a gradient of a growth factor is present at the wound edge. This phenomenon can only be studied by other researchers when they are willing to also use microfluidic technology. Finally, we show in chapter 7 that microfluidic technology is compatible with the widely used analysis technique of flow cytometry. By increasing compatibility between microfluidic technology and conventional technology, the threshold to employ microfluidics in cell biological research is lowered. This last point is important. The lack of compatibility between microfluidic technology and conventional laboratory equipment and tools does limit

the amount of information that can be deduced from an experiment. Virtually all analysis of experiments in microfluidic systems is performed by microscopy. Therefore, microfluidic experiments inherit the problems inherent to microscopy: the technique is labor-intensive and hardly quantitative. Increasing the amount of biochemical analysis tools that are compatible with microfluidcs is the main hurdle to take for microfluidic technology to become accepted by biologists. As described in chapter 7 of this thesis, there are two ways to overcome this problem of limited technology for biochemical analysis. Either the main biochemical analysis tools need to be scaled down so they can handle the small sample sizes in microfluidics, or microfluidic technology needs to be made compatible with conventional analysis tools. The former solution depends on long-term innovation and development of microfluidic devices, while the latter solution is more dependent on immediate creativity in adapting microfluidic devices or macroscopic assays in order to make them compatible with each other. When the connection between microfluidic devices and conventional analysis tools will have been established, nothing will stand in the way of microfluidics making its way into the field of cell biology.

So should biologists refrain from using microfluidic technology until this maturation has been realized? Of course not: the advantages are too big to ignore. And after all, realization of successful applications can be a major driving force for further development and improvement of the technology. It is the interaction between scientists from both fields, with the goal of increasing compatibility, on which the future success of microfluidic technology in cell biology depends.

Appendix: A microfluidic device for monitoring siRNA delivery under fluid flow

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When studying particle uptake in vitro, it is favorable to mimic the in vivo situation as much as possible. In this study we present a microfluidic device to mimic the mechanical stress caused by the flow of blood while studying particle uptake in vitro. Human endothelial cells were treated with liposomes containing fluorescent siRNA. It was found that applying physiologically relevant mechanical stress during transfection diminishes the uptake of liposomes in the cells.

Introduction

Particles for drug, gene or siRNA delivery are usually intended to deliver their cargo at specific sites of action, such as certain tissue types, or specific parts of the vasculature. In order to arrive at those sites of action, particles need to adhere to the target cells and they need to be endocytosed. Whether the particles adhere and are being taken up is dependent on the interplay of chemical and biological properties of the particles, the functional state of the endocytosing cell and the mechanical environment caused by the flow of blood. This is a complex process, involving biological, physical and chemical factors.[35] Therefore, it is recommendable to test for the ability of particles to successfully enter target cells. When studying the uptake of particles in vitro, usually the physiologically relevant stimulus of fluid flow is overlooked and endocytosis assays are carried out under static conditions. However, both in theory[35] and practice,[45] it has been shown that the mechanical stimulus of fluid flow is an important factor in the uptake of particles by cells. In order to screen quickly in the laboratory whether or not particles adhere to and are being taken up by cells under fluid flow conditions, a microfluidic approach is very useful: microfluidic devices are easy to handle, are good for direct microscopic imaging and use very small quantities of reagents. We have developed an easy-to-use microfluidic device that can be used to screen the uptake of particles under physiological fluid flow conditions. As a proof of principle, we show that the uptake of fluorescently labeled siRNA by human umbilical vein endothelial cells (HUVECs) is dependent on the fluid flow that is applied to the cells.

Methods

Microfluidic channels were created by pouring a silicone rubber pre-polymer (Sylgard 184) mixture on top of a silicon mold. After polymerization for 4 hours at 60 degrees Celsius, the resultant polymer slab (poly(dimethyl siloxane), PDMS) with the channel structures was peeled off of the silicon mold. The PDMS was then permanently bonded to a glass coverslip by treating both surfaces with oxygen plasma for one minute and pressing them together. The dimensions of the channels that were thus formed, were 60 µm × 500 µm × 2 cm (h × w × l). Holes were punctured in the PDMS to be able to reach the microfluidic channel. The microfluidic channels were coated with a fibronectin solution for two hours and washed with buffered salt solution. Then, a highly concentrated suspension of HUVECs (15 to 20 million cells per milliliter) was pipetted into the channel. A monolayer was allowed to form by incubating the cells overnight in the channel. The next day, microfluidic devices with HUVECs were ready for use in experiments of siRNA delivery under fluid flow, see also figure 31.

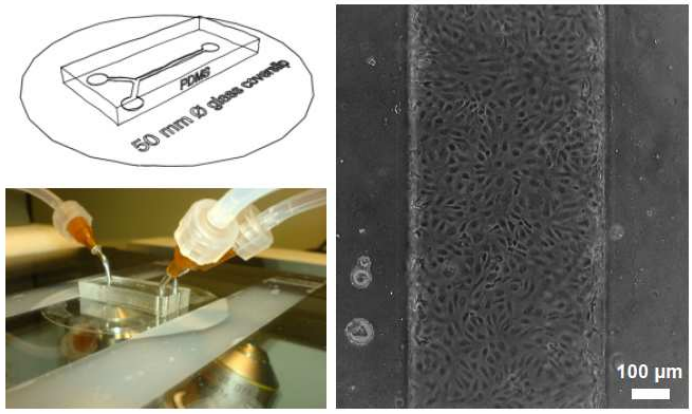

Figure 31: Top left: schematic depiction of the microfluidic device used in this study. Bottom left: microfluidic device connected to tubing and mounted on a heated microscope stage. Right: phase-contrast image of the human endothelial cell monolayer in the microfluidic channel.

In the experiment, the microfluidic devices were connected to a syringe pump using Tygon tubing and blunt needles. The endothelial monolayer was treated with siRNA-containing liposomes, formed by incubating FITC-labeled non-coding siRNA (Invitrogen) with the commercial transfectant oligofectamine (Invitrogen) in basal OptiMEM medium (Invitrogen). The liposomes were incubated with the cells for 2 hours, under static conditions, low fluid flow speed (20 µl/hour) or high fluid flow speed (1.5 ml/hour) in the microfluidic devices. These flow speeds theoretically generate shear stresses on the cells that are below the physiological range (0.01 Pa) and within the physiological range (1 Pa), respectively. After incubation under flow, the cells were washed with medium and incubated for 3 more hours in their normal growth medium (EGM-2, Lonza; without antibiotics). Subsequently, cells were washed with buffered salt solution and fixed with 4% paraformaldehyde. Nuclei were stained with DAPI. For imaging, the microfluidic devices were mounted on a Zeiss confocal laser scanning microscope and the fluorescent signals were detected, using the same settings for each

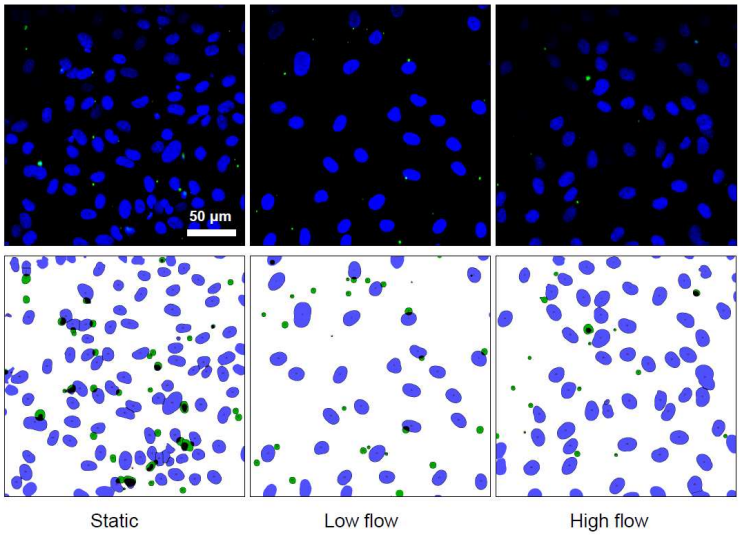

Figure 32: Top row: fluorescent images as acquired using confocal microscopy (40x objective). Nuclei are stained in blue, fluorescent siRNA appears in green. From left to right, images are from cells transfected under static conditions, low flow conditions and high flow conditions. Bottom row: image analysis. Nuclei are outlined in blue, green fluorescent spots are outlined in green, overlapping spots in black. Number of nuclei and green spots were determined per condition.

sample. Both nuclei and green fluorescent spots were automatically outlined and counted using ImageJ image analysis software.[1]

Results and discussion

We were able to successfully culture a confluent layer of endothelial cells in the PDMS microfluidic device (figure 31). Both in static conditions and under fluid flow, the cells could be transfected with liposomes containing fluorescently tagged siRNA, as indicated by the detection of bright green fluorescent spots in the cells (figure 32). By analyzing the amount of fluorescent spots relative to the amount of nuclei in each view, an estimation of transfection efficiency could be made. These first results show that uptake of the fluorescent siRNA is lower in cells that are treated with liposomes under high, physiologically relevant flow conditions compared to cells that are subjected to low flow or no flow at all (table 1). This is presumably due to the fact that liposome adhesion and uptake is countered by the mechanical stress caused by the fluid flow. Our results also show that low fluid flow conditions are more favorable for particle uptake than static conditions. This effect needs closer inspection, but this could be explained by a lowered particle concentration due to uptake under static conditions. The volume of particle mix present in the channel is so small (0.5 µl) that particle uptake by the cells can significantly lower the particle concentration in the transfection medium under static conditions, whereas the medium is constantly refreshed under flow. Therefore, comparing low flow and high flow conditions will probably yield the most reliable results.

Conclusion

We have shown that particle uptake under fluid flow conditions can be studied using a PDMS microfluidic device containing human endothelial cells. In this study,

Table 1: Summary of automated image analysis

	Static	Low shear	High shear
Number of nuclei	92	27	51
Number of spots	38	26	15
Spots per nucleus	0.41	0.96	0.29

we have shown the uptake of fluorescently labeled siRNA, but the set-up is suitable for studying uptake of any type of particle, as long as it is fluorescently tagged. In this proof-of-principle experiment, we have shown that the uptake of liposomes by endothelial cells is lowered when these cells are being subjected to physiological levels of mechanical stress. Therefore, it is recommendable to test in vitro the capacity of newly designed particles to be endocytosed by cells under mechanical stimulation. Testing this will probably increase the accuracy of predicting successful uptake of the particles in in vivo applications.

English and Dutch Summary

Author: A.D. van der Meer

English summary

Vascular endothelial cells form the inner lining of all blood vessels and are very important in regulating blood vessel function and preventing vascular diseases. Because endothelial cells are directly exposed to the flowing blood, they are constantly subjected to a tangential mechanical stress, or shear stress. Endothelial cells are highly responsive to this mechanical stimulus and exhibit a varied biological response when the magnitude or direction of the shear stress changes. Interestingly, major vascular diseases, like atherosclerosis, are predominantly localized in regions of the vascular tree with disturbed or turbulent blood flow patterns. In these regions, the endothelial cells are typically subjected to low shear stress or shear stress without a clear direction. The endothelial response to these shear stress patterns plays a role in the onset and development of disease. Therefore, the endothelial response to shear stress is an interesting object of research with a big clinical relevance.

When studying endothelial cells and their response to shear stress in the laboratory, relatively complicated and bulky set-ups are needed to exert physiologically relevant shear stress on the cells. Usually, these set-ups consist of two parallel plates with cells cultured onto them. Medium is then pumped through the space between the plates to simulate blood flow and exert shear stress. By scaling these set-ups down to micrometer-size, they become easier to handle and the flow rates that are needed to exert a physiologically relevant shear stress are several orders of magnitude lower. Moreover, smaller amounts of cells and reagents are needed to perform the same experiments. The manipulation of fluid in these micrometer-sized channels is an object of research in the field of microfluidics. An increasing amount of research is dedicated to applying microfluidic technology in cell biology.

The aim of the work that is described in this thesis is to generate more insight in the response of vascular endothelial cells to fluid shear stress by using microfluidic technology. This thesis shows a number of examples of successful applications of microfluidic technology for studying endothelial cell biology. By doing so, a number of unique advantages of microfluidic technology are highlighted.

In chapter 1, a general introduction to microfluidic technology, shear stress and endothelial cells is given. Moreover, the outline of the thesis is described in this chapter.

In chapter 2, an overview of the available literature on the application of microfluidic technology in vascular science is given. Most of the research has focused on the following subjects: the endothelial response to shear stress, assays for migration

of vascular cells, studying interactions between different vascular and blood cell types, controlled culturing of vascular cells in three dimensions, the screening of the effects of active compounds on vascular cells and the use of microfluidic technology for vascular tissue engineering. In this chapter, the research in this thesis is put in the proper perspective, by showing what other research themes are pursued in the field of microfluidics and vascular cell biology.

In Chapter 3 a 'conventional' study on the endothelial response to shear stress is described. It serves as a point of reference for the reader to understand what the advantages and limitations of the microfluidic approach are compared to the conventional approach. In the study in this chapter, some of the signal transduction pathways that are activated in the endothelial cell when it is subjected to shear stress are characterized. It is shown that initiation of signal transduction depends on the membrane scaffolding protein, caveolin-1. When this protein is downregulated by RNA interference, proper activation of the important signaling molecules protein kinase B (PKB)/Akt and extracellular-signal regulated kinase (ERK)1/2 is abrogated. This happens both when cells are subjected to shear stress and when they are subjected to vascular endothelial growth factor ($VEGF_{165}$). This finding suggests that the main function of caveolin-1 is to facilitate proper activation of the vascular endothelial growth factor receptor (VEGFR)-2. In this study, the activation status of proteins is screened by performing Western blotting of cell lysates and detecting phosphorylated forms of specific proteins. This analysis technique can not be applied in studies with microfluidic devices, because they simply do not contain enough cells. Therefore, new approaches for characterizing the endothelial response to shear stress need to be developed when transferring the research to micrometer-scales.

In Chapter 4, an example of such a microfluidic approach is given. Endothelial cells are cultured inside a 500 micrometer wide and 60 micrometer high channel. Then, they are subjected to a physiologically relevant shear stress. In response to this stimulus, endothelial cells reorganize their cytoskeleton to form actin fibers in the shear stress direction. By microscopic imaging of these cytoskeletal rearrangements and studying the directionality in the image, the degree of reorganization can be quantified. Then, when adding specific drugs to the medium, the signaling pathways that are important for mediating the shear stress signal to the imaged cytoskeletal effects can be identified. It is shown that when VEGFR-2 or p160Rho-associated kinase (p160ROCK) are inhibited, the process of reorganization is slowed down. On the other hand, inhibition of PKB/Akt has no effect. To sum up, it can be stated that microfluidic technology can be used to identify signaling events in response to shear stress, by using an approach that depends on microscopy.

Another study in which microfluidic technology and fluorescence microscopy are used to characterize an aspect of the endothelial response to shear stress is described

in Chapter 5. In this study, endothelial cells are ballistically injected with sub-micrometer-sized, fluorescent particles. These particles can be visualized with high-magnification, fluorescence microscopy and their motion can be tracked over time. Because the particles are embedded in the cytoskeletal mesh, their motion gives information on the cytoskeletal micromechanics. When endothelial cells are subjected to shear stress, the motion of the embedded particles decreases rapidly, but transiently: within ten minutes after the onset of shear stress, the particle motion is at a minimum, and after fifteen to thirty minutes it has returned to control values. These changes in particle dynamics are depended on activation of VEGFR-2, because the decrease in particle motion is not observed in the presence of an inhibitor of VEGFR-2. Together with the findings in chapter 4, this shows that VEGFR-2 is an essential component in both the prolonged, as well as the fast and transient, cytoskeletal changes that occur in endothelial cells in response to shear stress.

Chapter 6 deals with the effects of shear stress on endothelial cell migration. In this chapter, a microfluidic version of the endothelial wound healing assay is described. The wound healing assay works by culturing a monolayer of endothelial cells in a microfluidic channel and flushing the channel with three parallel fluid flows, of which the middle one contains the protease trypsin. As a result, cells in the middle of the channel are detached and flushed away, leaving an artificial wound in the endothelial monolayer. This wound heals over time by endothelial cells that migrate into the open area. When, during this process of migration, the cells are treated with shear stress or a growth factor, the wound healing rate increases. A unique advantage of the microfluidic assay is that a stable growth factor gradient can also be applied to the edges of the wound. It is shown that application of such a gradient significantly increases the wound healing rate compared to application of a single growth factor concentration.

In summary, in chapters 4, 5 and 6 a number of successful applications of microfluidic technology for studying the endothelial response to shear stress are described. Chapter 7 focuses on the limitations of the technology. The major limitation of microfluidic technology is its incompatibility with conventional analysis tools. An example is the study in chapter 3, which could never have been performed with microfluidic technology, because microfluidic set-ups do not harbor enough cells to be analyzed with Western blotting. Flow cytometry, another major conventional analysis tool, was also considered to be incompatible with microfluidic devices. The study in chapter 7 shows that properly designed microfluidic devices do contain enough cells to be reliably analyzed with flow cytometry. In order to show this, the uptake of fluorescent low density lipoprotein (LDL) by endothelial cells that are cultured in a microfluidic device is followed by analysis with flow cytometry. This uptake increases linearly over the time course of at least one hour. Moreover, it is found that when

endothelial cells are pre-treated with shear stress, the uptake of LDL is decreased. Compared to analysis by fluorescence microscopy, the method described in this chapter is a lot faster and more quantitative.

Finally, in chapter 8, it is concluded that microfluidic technology can be employed to develop useful tools for studying the endothelial cell response to shear stress in vitro. Still, the technology is not widely accepted by cell biologists. This is mainly due to the limited compatibility with conventional analysis tools. If this challenge can be overcome, nothing stands in the way of microfluidic devices to become widely used tools in vascular cell biology.

In the Appendix, an exploratory study for developing an assay to follow the uptake of short interfering RNA by endothelial cells under conditions of fluid flow is described. It is shown that microfluidic devices are ideally suited for performing these types of assay, because of their low consumption of cells and reagents and their potential for increasing throughput by parallelization. In the future, this application of microfluidic technology may also be developed further.

Nederlandse samenvatting

Vasculaire endotheelcellen vormen de binnenbekleding van alle bloedvaten en zijn zeer belangrijk in het regelen van bloedvatfunctie en in het voorkomen van vataandoeningen. Omdat de endotheelcellen in direct contact staan met het stromende bloed, worden ze constant blootgesteld aan een mechanische spanning aan het oppervlak, de schuifspanning. Endotheelcellen reageren sterk op deze mechanische stimulus en vertonen een veelzijdige biologische respons wanneer de grootte of de richting van de schuifspanning verandert. Interessant genoeg zijn belangrijke vataandoeningen, zoals atherosclerose, vooral te vinden in delen van het vaatstelsel met verstoorde of turbulente patronen van bloedstroom. In deze gebieden worden de endotheelcellen veelal blootgesteld aan lage schuifspanning of schuifspanning zonder duidelijke richting. De respons van de endotheelcellen op deze patronen van schuifspanning speelt een rol in de aanzet en ontwikkeling van aandoeningen. Zodoende is de respons van endotheelcellen op schuifspanning een interessant onderzoeksonderwerp met grote klinische relevantie.

Wanneer endotheelcellen en hun respons op schuifspanning in het laboratorium worden bestudeerd, zijn er grote en relatief complexe opstellingen nodig om een fysiologisch relevante schuifspanning uit te oefenen op de cellen. Vaak bestaan deze opstellingen uit twee parallele platen waarop de cellen zijn gekweekt. Vervolgens wordt er medium tussen de platen door gepompt om een bloedstroom te simuleren en schuifspanning uit te oefenen. Wanneer deze opstellingen verkleind worden tot op micrometerschaal, worden deze eenvoudiger te hanteren en zijn de stroomsnelheden die nodig zijn om fysiologisch relevante schuifspanningen op te wekken vele

grootteordes lager. Bovendien zijn er kleinere hoeveelheden cellen en reagentia nodig om dezelfde experimenten uit te voeren. Manipulatie van vloeistof in deze kanalen op micrometerschaal is een onderzoeksobject in het veld van de microfluidica. Er wordt steeds meer onderzoek gedaan naar het toepassen van microfluidische technologie in de vasculaire celbiologie.

Het doel van het werk dat wordt beschreven in dit proefschrift is om meer inzicht te verkrijgen in de respons van endotheelcellen op schuifspanning door het gebruik van microfluidische technologie. Dit proefschrift beschrijft een aantal voorbeelden van de succesvolle toepassing van microfluidische technologie in het onderzoeken van endotheelcelbiologie. Door het beschrijven van deze toepassingen worden een aantal unieke voordelen van microfluidische technologie uitgelicht.

In Hoofdstuk 1 wordt een algemene inleiding op microfluidische technologie, schuifspanning en endotheelcellen gegeven. Verder wordt er de lijn van het proefschrift beschreven.

In Hoofdstuk 2 wordt een overzicht gegeven van de beschikbare literatuur over de toepassing van microfluidische technologie in vasculair wetenschappelijk onderzoek. Het meeste onderzoek richt zich op de volgende onderwerpen: de respons van endotheelcellen op schuifspanning, tests voor het volgen van migratie van vasculaire cellen, het bestuderen van interacties tussen verschillende types bloedcellen en vasculaire cellen, het gecontroleerd kweken van vasculaire cellen in drie dimensies, het in kaart brengen van de effecten van actieve stoffen op vasculaire cellen en het toepassen van microfluidische technologie in de vasculaire weefseltechnologie. In het hoofdstuk wordt het onderzoek in dit proefschrift in een juist perspectief geplaatst door te laten zien welke andere onderzoeksthema's worden beoefend in het veld van microfluidica en vasulaire celbiologie.

In Hoofdstuk 3 wordt een 'conventionele' studie naar de respons van endotheelcellen op schuifspanning beschreven. Het dient als een referentiepunt voor de lezer om te begrijpen wat de voordelen en beperkingen zijn van een microfluidische aanpak in vergelijking met een conventionele aanpak. In de studie in dit hoofdstuk worden enkele van de signaaltransductieroutes die geactiveerd worden in endotheelcellen wanneer zij worden blootgesteld aan schuifspanning in kaart gebracht. Er wordt aangetoond dat het op gang komen van deze signaaltransductie afhangt van een membraangeassocieerd, structureel eiwit, caveolin-1. Wanneer de expressie van dit eiwit wordt verlaagd door middel van RNA interferentie, wordt de juiste activatering van de belangrijke signaaltransductiemoleculen protein kinase B (PKB)/Akt en extracellular-signal regulated kinase (ERK)1/2 teniet gedaan. Dit gebeurt zowel wanneer cellen worden blootgesteld aan schuifspanning, als wanneer zij worden behandeld met vascular endothelial growth factor ($VEGF_{165}$). Deze bevinding suggereert dat de belangrijkste functie van caveolin-1 het vergemakkelijken van de activatie van vascular

endothelial growth factor receptor (VEGFR)-2 is. In deze studie wordt de activatiestatus van eiwitten onderzocht door cellysaten te onderwerpen aan Western blotting en de gefosforyleerde vorm van specifieke eiwitten te detecteren. Deze analysetechniek kan niet worden toegepast in studies met microfluidische hulpmiddelen, omdat deze simpelweg niet genoeg cellen bevatten. Daarom moet er een nieuwe aanpak worden ontwikkeld wanneer het onderzoek naar de respons van endotheelcellen op schuifspanning wordt overgebracht naar micrometerschaal.

In Hoofdstuk 4 wordt een voorbeeld van zo'n aanpak gegeven. Endotheelcellen worden gekweekt in een kanaal dat 500 micrometer breed is en 60 micrometer hoog. Vervolgens worden zij blootgesteld aan een fysiologisch relevante schuifspanning. In respons op deze stimulus reorganiseren de endotheelcellen hun celskelet om actindraden te vormen in de richting van de schuifspanning. Door deze herschikking van het celskelet microscopisch in beeld te brengen en de richting van het beeld te bestuderen, kan de mate van reorganisatie worden gekwantificeerd. Wanneer er vervolgens specifieke actieve stoffen worden toegevoegd aan het medium, kunnen de signaaltransductieroutes die belangrijk zijn voor de in beeld gebrachte effecten op het celskelet worden geïdentificeerd. Er wordt aangetoond dat wanneer VEGFR-2 of p160Rho-associated kinase (p160ROCK) geremd worden, het reorganisatieproces vertraagd is. Aan de andere kant heeft het remmen van PKB/Akt geen effect. Samenvattend kan er gesteld worden dat microfluidische technologie gebruikt kan worden voor het in kaart brengen van signaaltransductie in respons op schuifspanning door het gebruik van een aanpak die steunt op microscopie.

Een andere studie waarin microfluidische technologie en fluorescentiemicroscopie gebruikt worden om één van de aspecten van de respons van endotheelcellen op schuifspanning te karakteriseren is beschreven in Hoofdstuk 5. In deze studie worden endotheelcellen ballistisch geïnjecteerd met fluorescente deeltjes van kleiner dan een micrometer. Deze deeltjes kunnen zichtbaar gemaakt worden met fluorescentiemicroscopie bij een hoge vergroting en hun bewegingen kunnen over de tijd gevolgd worden. Omdat de deeltjes zijn ingebed in het netwerk van het celskelet, geeft hun beweging informatie over de micromechanica van het celskelet. Wanneer endotheelcellen worden blootgesteld aan schuifspanning, neemt de beweging van de deeltjes snel, maar kortstondig, af: binnen tien minuten na het begin van de schuifspanning is de beweging van de deeltjes minimaal, en na vijftien tot dertig minuten is de beweging terug op het niveau van de controlesituatie. Deze veranderingen in de dynamica van de deeltjes zijn afhankelijk van de activering van VEGFR-2, omdat de vermindering van de beweging van de deeltjes niet wordt waargenomen in de aanwezigheid van een VEGFR-2 remmer. Samen met de bevindingen in hoofdstuk 4 toont dit aan dat VEGFR-2 een essentieel onderdeel is in zowel de lange als de snelle en kortston-

dige veranderingen in het celskelet die plaatsvinden in endotheelcellen in respons op schuifspanning.

Hoofdstuk 6 behandelt de effecten van schuifspanning op endotheelcelmigratie. In dit hoofdstuk wordt een microfluidische versie van de wondgenezingstest voor endotheelcellen beschreven. De wondgenezingstest werkt door een laag endotheelcellen in een microfluidisch kanaal te kweken en het kanaal door te spoelen met drie parallele vloeistofstromen, waarvan de middelste het protease trypsin bevat. Als gevolg hiervan worden de cellen in het midden van het kanaal losgemaakt en weggespoeld, waardoor een kunstmatige wond in de laag cellen achterblijft. Deze wond geneest over de tijd doordat endotheelcellen het open gebied in migreren. Wanneer tijdens dit proces de cellen behandeld worden met schuifspanning of een groeifactor, neemt de snelheid van wondgenezing toe. Een uniek voordeel van de microfluidische test is dat er ook een stabiele gradiënt van een groeifactor kan worden aangelegd op de wondrand. Er wordt getoond dat het aanleggen van een dergelijke gradiënt de snelheid van wondgenezing significant doet toenemen in vergelijking met een enkele groeifactorconcentratie.

Kort samengevat, in de hoofdstukken 4, 5 en 6 worden een aantal succesvolle toepassingen van microfluidische technologie voor het bestuderen van de respons van endotheelcellen op schuifspanning beschreven. Hoofdstuk 7 is gericht op de beperkingen van de technologie. De belangrijkste beperking van microfluidische technologie is het gebrek aan compatibiliteit met conventionele gereedschappen voor analyse. Een voorbeeld is de studie in hoofdstuk 3 die nooit had kunnen worden uitgevoerd met behulp van microfluidische technologie, omdat microfluidische opstellingen niet genoeg cellen herbergen om te worden geanalyseerd met Western blotting. Flowcytometrie, nog een belangrijk gereedschap voor analyse, werd ook beschouwd als niet compatibel met microfluidische hulpmiddelen. De studie in hoofdstuk 7 toont aan dat op de juiste manier ontworpen microfluidische hulpmiddelen genoeg cellen bevatten om deze op een betrouwbare manier te analyseren met flowcytometrie. Om dit aan te tonen, wordt de opname van low density lipoprotein (LDL) door endotheelcellen die in een microfluidisch kanaal zijn gekweekt gevolgd door middel van flowcytometrie. Deze opname neemt lineair toe over een tijdspanne van ten minste één uur. Bovendien wordt gevonden dat de opname van LDL vermindert wanneer de endotheelcellen voorbehandeld zijn met schuifspanning. Vergeleken met analyse door fluorescentiemicroscopie, is de methode die in dit hoofdstuk beschreven wordt een stuk sneller en meer kwantitatief.

Ten slotte wordt er in Hoofdstuk 8 geconcludeerd dat microfluidische technologie gebruikt kan worden om nuttige gereedschappen te ontwikkelen voor het bestuderen van de in vitro respons van endotheelcellen op schuifspanning. Toch wordt de technologie niet breed geaccepteerd door biologen. Dit is voornamelijk te wijten aan

het gebrek aan compatibiliteit met conventionele analysetechnieken. Wanneer deze moeilijkheid overwonnen kan worden, staat niets de microfluidische technologie nog in de weg om op brede schaal te worden gebruikt in de vasculaire celbiologie.

In de Appendix wordt een verkennende studie beschreven naar het ontwikkelen van een test om de opname van short interfering RNA door endotheelcellen onder omstandigheden van vloeistofstroom te volgen. Er wordt aangetoond dat microfluidische hulpmiddelen ideaal zijn voor het uitvoeren van een dergelijke test, vanwege het lage verbruik van cellen en reagentia en de mogelijkheid tot opschalen door het paralleliseren van de test. Deze toepassing van microfluidische technologie zou in de toekomst ook verder ontwikkeld kunnen worden.

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