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

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The physiology of vascular endothelial cells is strongly affected by fluid shear stress on their surface. In this study, a microfluidic assay was employed to analyze the alignment of actin filaments in endothelial cells in response to shear stress. When cells were cultured in microfluidic channels and subjected to shear stress, the alignment of filaments in the channel direction was significantly higher than in static cultures. By adding inhibitory drugs, the roles of several signaling proteins in the process of alignment were determined. Thus, it is shown how microfluidic technology can be employed to provide a mechanistic insight into cell physiology. © *2010 American Institute of Physics.* [doi:10.1063/1.3366720]

I. INTRODUCTION

Vascular endothelial cells form the inner lining of all blood vessels. These cells have an important role in blood vessel function and disease.[1] 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.[2]

Because vascular disease is predominantly localized to regions with abnormal shear stress patterns,[3] 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.

Recently, microfluidic setups for subjecting endothelial cells to shear stress *in vitro* have been reported.[4,5] Micrometer-sized channels were produced by 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 offers a number of advantages over the conventional macroscopic setups. For example, smaller amounts of cells and reagents are needed per experiment and very low flow rates can be used to exert physiological levels of shear stress.[4,5] Moreover, microfluidic devices can incorporate multiple parallel channels for applying a range of different shear stress magnitudes.[4]

So far, the reports of these microfluidic assays have mainly focused on proof of principle and on reproduction of well-established facts in vascular literature.[2,4,6] However, for microfluidic tools to become more widely accepted by biologists, it is important to apply microfluidic technology for gaining cell biological knowledge. In this study, we show an example of this approach by analyzing the shear stress-induced alignment of actin filaments in the absence and presence of several inhibitors.

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II. MATERIALS AND METHODS

A. Device fabrication

Microfluidic channels were prepared by pouring polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, USA) onto a silicon wafer with rectangular SU-8 photoresist structures (100 μm wide, 120 μm high, and 2 cm long). After cross-linking of the PDMS, the surfaces of the PDMS devices and of glass cover slips were activated by treatment in an oxygen plasma sterilizer. Irreversible bonding between the two was accomplished by pressing them together.

B. Cell culture

Endothelial cells were isolated from human umbilical cord veins[7] and were cultured with endothelial growth medium-2 (Lonza, The Netherlands) in polystyrene flasks, coated with 2 mg/ml fibronectin (Sanquin, Amsterdam, The Netherlands). For shear stress experiments, a cell suspension of 20×10^6 cells/ml was pipetted into a fibronectin-coated channel. After incubation for 6 h, channels were flushed once to remove any nonattached cells.

C. Shear stress application

The endothelial cell-covered microfluidic channels were connected to a syringe pump using Tygon tubing (Rubber BV, The Netherlands) and blunt needle connections (EFD, Inc., USA). The shear stress medium was medium 199 (Lonza, The Netherlands), containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and in some experiments, a specific inhibitor [10 μM Y-27632 (Sigma, Germany) for p160-Rho-associated kinase/p160ROCK, 5 μg/ml vascular endothelial growth factor (VEGF) receptor-2/VEGFR-2 inhibitor (CalBiochem, USA), or 5 μg/ml LY294002 (Sigma, Germany) for phosphoinositide 3-kinase/PI3K]. The medium was pumped through the channels at a rate of 1.5 ml/h for 16 h. The average wall shear stress was calculated to be approximately 1.5 Pa, using the following equation: $\tau=6Q\mu/w^2h$, with τ as the shear stress in Pa, Q as the flow rate in m^3/s, μ as the dynamic viscosity in Pa s, and w and h as the width and height of the channel in meters, respectively.

D. Actin filament analysis

After shear stress application, cells were fixed with 4% paraformaldehyde and the actin filaments were labeled with 1 μg/ml phalloidin-fluorescein isothiocyanate (Invitrogen, USA). The staining pattern was imaged on a Zeiss LSM510 confocal fluorescence microscope.

For each channel, images of ten different locations were taken and analyzed. All image analysis was performed with NIH IMAGEJ software,[8] using a method that has been used before for determining directionality in collagen fibers.[9] For a visualization, see Fig. 1. First, a 65×65 μm^2 region was selected from the middle of the channel to limit wall effects. The resulting image was transformed to the frequency domain by performing a fast Fourier transform (FFT). The transformed image was contrasted to exclude all low intensity, high frequency signal. The remaining high amplitude, low frequency waveforms represent the general structures in the original picture. Total pixel intensities for 18 10° categories in the frequency domain image were calculated. The resulting values were normalized to percentages of total pixel intensity. 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, the peak percentage was converted into an alignment index. An index of 0 corresponds to an even distribution of pixel intensities over all 10° categories (no alignment), while an index of 1 corresponds to a distribution with all pixel intensities in the 30° peak (complete alignment).

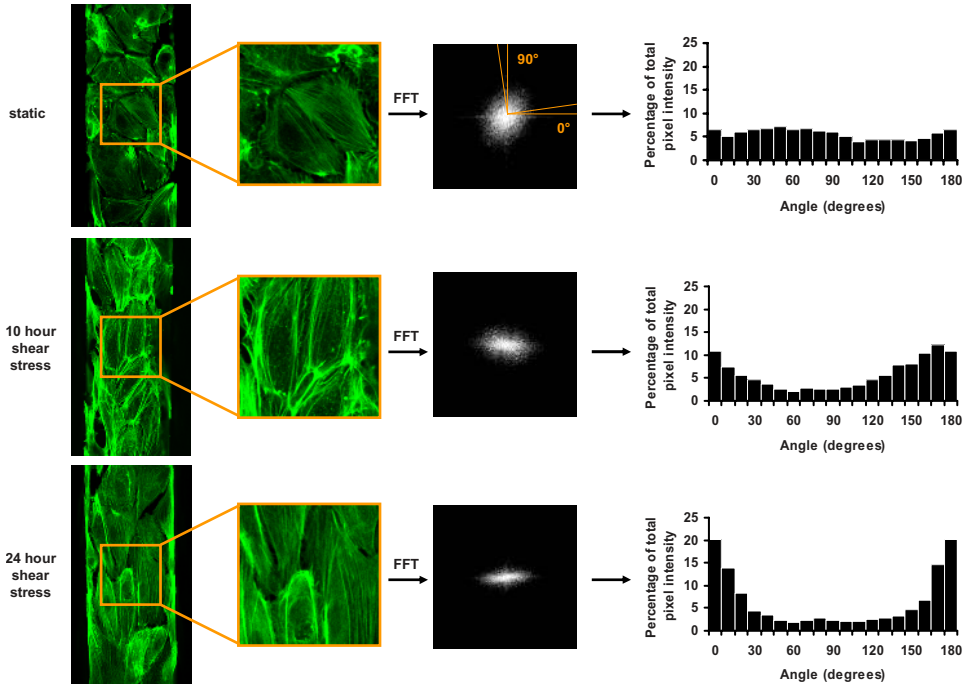

FIG. 1. Method for determining actin filament directionality by frequency domain analysis. Endothelial cells were cultured in microfluidic channels, treated with 1.5 Pa shear stress for several periods and then fixed and stained for actin filaments (left). Subsequently, 65×65 μm^2 image cutouts were taken from the middle of the channel. These cutouts were automatically transformed to the frequency domain by performing a FFT. The actin filament structures in the original picture can be considered as a pattern of interfering waves of different intensities, frequencies, and directions. 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° 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.

E. VEGF treatment

Before treating cells with VEGF, they were starved for 2 h in endothelial basal medium-2 (Lonza, The Netherlands) containing 10 mg/ml bovine serum albumin. After starvation, some wells were pretreated with 5 μg/ml LY294002 for 1 h. Then, the cells were stimulated for 1 h with 10 ng/ml VEGF.

III. RESULTS AND DISCUSSION

Endothelial cells were subjected to shear stress for 16 h in microfluidic channels. First of all, the alignment indexes of statically cultured cells and cells that had been subjected to shear stress were compared. As expected, the index of sheared samples (0.19 ± 0.04) was significantly higher (student's t-test, $p < 0.001$) than the index of statically cultured samples (0.06 ± 0.02).

Subsequently, the effects of several inhibitors on the shear stress-induced cytoskeletal rearrangements were tested. First of all, when p160ROCK was inhibited, the shear stress-induced alignment was absent (Fig. 2). p160ROCK is a downstream target of the small GTPase Rho[10] and is responsible for enhancing myosin light chain activation. Myosin light chain is a component of the filamentous actin network and its activation is responsible for the formation of actin stress fibers.[10]

Second of all, when VEGFR-2 was inhibited during shear stress application, the normally observed alignment was lowered (Fig. 2). VEGFR-2 is part of a mechanosensory complex. Cells that lack one of the components of this complex do not align in the direction of flow.[11] Our results

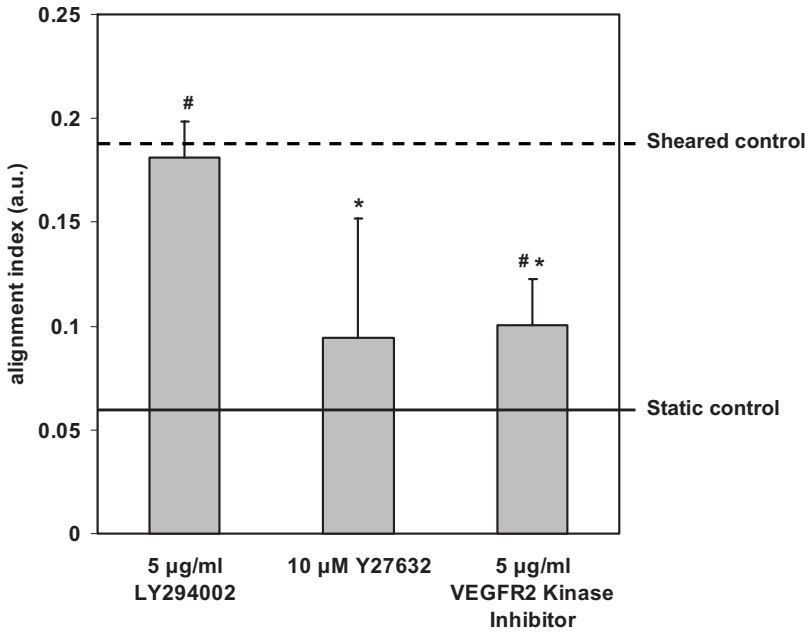

FIG. 2. The effect of specific inhibitors on the alignment index in sheared samples. Endothelial cells were treated with 1.5 Pa shear stress for 16 h in the presence of specific inhibitors of PI3K (LY294002), p160ROCK (Y27632), and VEGFR-2 (VEGFR-2 kinase inhibitor). The dashed line represents the average alignment index of sheared samples without the inhibitors (n=4). The solid line denotes the alignment index in statically cultured cells (n=5). 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, $p < 0.05$).

confirm that VEGFR-2 is essential for cytoskeletal remodeling in response to shear stress. However, which pathway mediates 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 because shear stress induces VEGFR-2-dependent PI3K/Akt signaling.[11] This signaling is responsible for both the shear stress-induced production of nitric oxide and the resistance to apoptosis of sheared endothelial cells.[12]

However, inhibition of PI3K had no significant effect on shear stress-induced alignment (Fig. 2). In order to show that the absence of an effect was not caused by ineffectiveness of the inhibitor, endothelial cells were treated with VEGF in the presence of the inhibitor. The inhibitor was able to block cytoskeletal remodeling in response to this growth factor (Fig. 3), demonstrating its effectiveness. The finding that PI3K/Akt signaling is not involved in shear stress-induced alignment of endothelial cells is surprising because it is a major pathway in shear stress signaling and it is involved in cytoskeletal remodeling in response to other stimuli (e.g., the VEGF treatment shown in this and other studies[13]). So far, there has been only one study in which the role of this pathway in the cytoskeletal remodeling of shear stress-treated cells was investigated.[14] That study showed that PI3K signaling does not have an impact on actin dynamics in single endothelial cells that migrate along with the direction of shear stress. Together with our current findings on cytoskeletal remodeling in confluent cells, this suggests that another pathway is involved in inducing cytoskeletal changes in response to shear stress. The most logical candidate for this pathway is integrin signaling. Activation of these transmembrane receptors in response to shear stress is dependent on VEGFR-2 and is necessary for activation of the small GTPase Rho.[11,15] This would explain our findings that VEGFR-2 and p160ROCK, but not PI3K, are needed for actin alignment in response to shear stress.

To our knowledge, this study is the first in which microfluidic technology is applied to

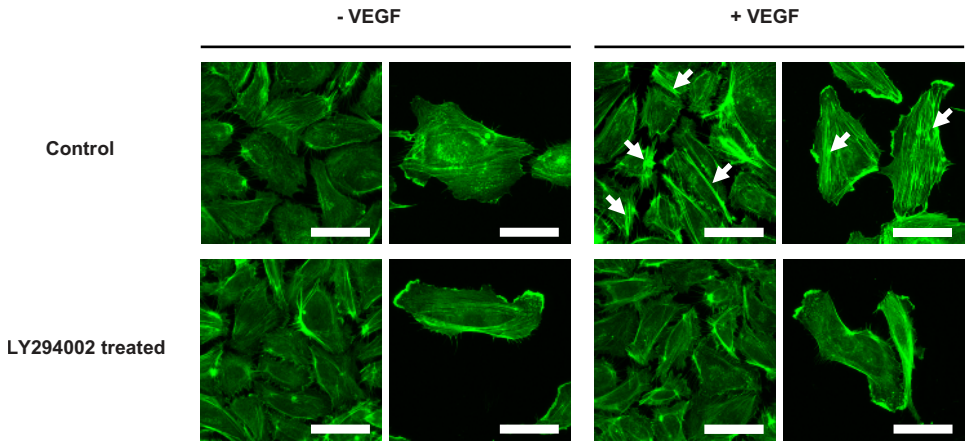

FIG. 3. The effect of VEGF treatment and inhibition of the PI3K/Akt pathway on stress fiber formation. In the absence of VEGF, endothelial cells have thin actin filaments running through the cell body (top left). When treated for 1 h with 10 ng/ml VEGF (top right), these fibers increase in number and thickness. This effect is absent in cells that have been pretreated with an inhibitor for PI3K (bottom). Arrows denote the location of thick actin filament bundles. Scale bars, 50 μm.

generate new biological insight into the endothelial response to shear stress. We hope that our approach in this study serves as an example on how to apply microfluidic technology for generating biological knowledge.

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