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Human brain microvascular endothelial cells resist elongation due to shear stress

Adam Reinitz [1], Jackson DeStefano [1], Mao Ye, Andrew D. Wong, Peter C. Searson *

[a] Department of Materials Science and Engineering, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA
[b] Institute for Nanobiotechnology, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA

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ABSTRACT

Endothelial cells in straight sections of vessels are known to elongate and align in the direction of flow. This phenotype has been replicated in confluent monolayers of bovine aortic endothelial cells and human umbilical vein endothelial cells (HUVECs) in cell culture under physiological shear stress. Here we report on the morphological response of human brain microvascular endothelial cells (HBMECs) in confluent monolayers in response to shear stress. Using a microfluidic platform we image confluent monolayers of HBMECs and HUVECs under shear stresses up to 16 dyne cm^{-2}. From live-cell imaging we quantitatively analyze the cell morphology and cell speed as a function of time. We show that HBMECs do not undergo a classical transition from cobblestone to spindle-like morphology in response to shear stress. We further show that under shear stress, actin fibers are randomly oriented in the cells indicating that there is no cytoskeletal remodeling. These results suggest that HBMECs are programmed to resist elongation and alignment under shear stress, a phenotype that may be associated with the unique properties of the blood–brain barrier.

Introduction

Blood flow results in a frictional drag, or shear stress, on the endothelial lining of vessel walls parallel to the direction of flow. These stresses play an important role in regulating endothelial cell morphology and function, and in mediating a wide range of signaling and transport processes between the vascular system and surrounding tissue (Aird, 2007a, 2007b; Chien, 2007; Davies, 1995; Johnson et al., 2011).

Endothelial cells in straight sections of large resected vessels and away from branch points exhibit an elongated, spindle-like morphology (Davies, 1995; Dolan et al., 2013; Kibria et al., 1980; Levesque et al., 1986; Nerem et al., 1981; Reidy and Langille, 1980; Silkworth and Stehbens, 1975; Zand et al., 1988). When subjected to a physiological shear stress in 2D cell culture, confluent monolayers of bovine aortic endothelial cells (BAEs), human umbilical vein endothelial cells (HUVECs), and primary baboon artery endothelial cells (BAECs) undergo a transition from a cobblestone morphology to an elongated spindle-like morphology and align in the direction of flow (Blackman, 2002; Chien, 2007; Chiu et al., 1998; Davies, 1995; Ensley et al., 2012; Eskin et al., 1984; Levesque and Nerem, 1985, 1989; Malek and Izumo, 1996; Simmers et al., 2007). A similar morphological response has been reported for human abdominal aortic endothelial cells seeded onto the inner surface of a polydimethyl siloxane tube (Farcas et al., 2009; Rouleau et al., 2010). The response of BAEs and HUVECs to shear stress results in a morphology similar to that of endothelial cells in resected vessels, which provides evidence that mechano-transduction modulates cellular function and is important in maintaining vascular homeostasis (Chien, 2007; Johnson et al., 2011).

Morphological parameters associated with endothelial cells in confluent monolayers in response to shear stress and resected vessels are summarized in Table 1. Endothelial cells in straight sections of the aorta across several animal species are characterized by an inverse aspect ratio (IAR, cell width/cell length) of about 0.20, a circularity of about 0.3, and an average orientation angle with respect to the flow direction (θ) of 5–15°. Similar morphological parameters have been reported for 2D confluent monolayers of BAEs and HUVECs in cell culture under shear stress. The somewhat larger variability in morphological parameters seen in cell culture is due in part to the different experimental conditions and the fact that the morphology is often characterized at a single time point.

In previous work we have reported on the influence of curvature on the morphology of endothelial cells. By seeding confluent monolayers of endothelial cells on collagen-coated glass rods of different diameters, we studied the influence of curvature on endothelial cell morphology (Ye et al., 2014). To minimize the effects of curvature, HUVEC cells elongate and align in the axial direction with decreasing diameter. In contrast, human brain microvascular endothelial cells (HBMECs) do not elongate or align in the axial direction but wrap around in the radial direction with little change in morphology as the diameter decreases

* Corresponding author.
E-mail address: searson@jhu.edu (P.C. Searson).
[1] Contributed equally.

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(Ye et al., 2014). The endothelial cells in the brain microvasculature are highly specialized, with an array of transporters, efflux pumps, and tight junctions that are an important component of the blood–brain barrier, regulating transport into and out of the brain (Wong et al., 2013). These results suggest that HBMECs may also display a unique morphological phenotype.

Elongation and alignment in response to shear stress is thought to be a universal phenotype of endothelial cells. However, our previous work suggests that brain microvascular endothelial cells may be programmed to respond differently to physical stimuli, such as curvature, compared to endothelial cells from larger vessels. Therefore, here we compare the morphological response of HBMECs, representative of brain capillaries, and HUVECs, representative of large vessels, to shear stress. We show that HBMECs do not elongate and align in response to physiological shear stress. In addition, we show that actin fibers are randomly oriented within HBMECs and do not align with flow. These results suggest that HBMECs are programmed to resist elongation and alignment in response to shear stress. This phenotype may be associated with the unique properties of the blood–brain barrier.

Materials and methods

Microfluidic platform

The microfluidic device (Fig. 1a–b) was fabricated from polydimethylsiloxane (PDMS, Sylgard) using a machined aluminum mold with four rectangular channels connected in series. Each channel was 4 mm wide and 50 mm long, with heights of 390 μm, 450 μm, 550 μm, and 770 μm, respectively. PDMS was poured to fill half of the mold and partially cured at 100 °C for 15 min. Nylon spacers (5 mm ID, McMaster) forming the bubble traps were placed on top of the PDMS and sealed with a second layer of PDMS cured at 100 °C for 45 min. The PDMS blocking the bubble traps was removed using a 5 mm inner diameter hole punch and the inlets and outlets were made using a 1.50 mm hole punch. The PDMS channels were then plasma bonded to a 50 mm × 75 mm glass microscope slide (Corning). 6.35 mm ID silicon tubing (McMaster) was used to connect the nylon inserts to the caps of the bubble traps, which was a male Luer to hosebarb connector with a female Luer cap (Cole Parmer).

For Poiseuille flow in a rectangular channel, the wall shear stress was given by $\tau = 6Q\mu/h^2w$ where Q was volumetric flow rate, μ was dynamic viscosity, h was channel height, and w was channel width. The channel heights were chosen such that the shear stress in the four channels scales in the ratio 1:2:3:4.

The flow setup was composed of a custom machined Teflon media reservoir connected to 1/8″ ID silicon tubing (McMaster) (Fig. 1a–c). Media from the Teflon reservoir passed through a 1.5 m coil of silicone tubing located in a gas exchange chamber (In Vivo Scientific) of humidified 5% CO_2. The flow was then directed through the microfluidic device and returns to the media reservoir via the programmable peristaltic pump (New Era Pumps, NE-9000). Teflon tubing was used for the return flow from the peristaltic pump to the media reservoir. The peristaltic pump was programmed to gradually increase flow stepwise from 1.25 to 7.5 mL min^{-1} over the first 6 h, increasing by 1.25 mL min^{-1} every hour. After the 6 h ramp up, the flow was maintained at 10 mL min^{-1} which equates to a time average shear stress of 4, 8, 12, and 16 dyne cm^{-2} within four channels respectively. Flow was applied to HBMECs for 36 h and HUVECs for 72 h. Characterization of the flow produced from the peristaltic pump confirms it was pulsatile (see Supplementary Information). The flow rates were verified using a flow meter (Liquid Flowmeter NIST-traceable calibration, Cole Parmer) that measured the output from the microfluidic device to verify the time averaged flow rate. The flow profile was verified by measuring the velocity of fluorescent beads at different heights in the channels (see Supplementary Information for details). For static experiments, cells were seeded into a device and allowed to grow to confluence for 24 h. To avoid depletion of nutrients, the media was briefly circulated approximately every 8 h.

Cell culture

Immortalized human brain microvascular endothelial cells (HBMECs) (Nizet et al., 1997) were cultured in M199 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 1% penicillin streptomycin (Invitrogen). In a comparison of four immortalized human brain microvascular endothelial cell lines, this cell line showed the highest transendothelial electrical resistance values and was determined to be the most suitable for an *in vitro* blood–brain barrier model (Eigenmann et al., 2013). Human umbilical vein endothelial cells (HUVECs) (Promocell, Heidelberg, Germany) were grown in endothelial cell growth medium (EGM-2, Promocell) containing endothelial basal medium (EBM), 2% fetal calf serum (FCS), and 1% penicillin streptomycin, hEGF, hydrocortisone, VEGF, hbFGF, R3 IGF, AA-500, and Heparin. Both cell lines were cultured under physiological conditions on uncoated tissue culture polystyrene flasks (Sarstedt).

Before introducing the cells into the microfluidic device, cells were thoroughly washed twice with PBS without Ca^{2+} or Mg^{2+} (Lonza) and removed from their culture surface using 0.5% EDTA/trypsin (Invitrogen) for 3 min at 37 °C. Prior to seeding cells, the interior walls of the channels were coated with 62.5 μg mL^{-1} fibronectin (BD Biosciences, San Jose, CA) for 1 h at room temperature. HBMECs and HUVECs were introduced at concentrations of 1,500,000 cells mL^{-1} and 2,000,000 cells mL^{-1}, respectively, and grown to confluence in their respective culture media. Each channel was seeded with 100 μL of cell suspension, resulting in 150,000 cells for HBMEC channels and 200,000 cells for HUVEC channels. Prior to applying shear stress, the media was changed to reduced growth factor media composed of EBM supplemented with 2% FCS. The microfluidic device was mounted in a live-cell chamber (In Vivo Scientific) on the microscope, maintained at 37 °C and 5% CO_2. Static experiments were conducted using a similar procedure. Cells were seeded into a device and allowed to grow to confluence for 24 h. At confluence, the flow loop was connected and the media changed to reduced growth factor media. Approximately every 8 h the pump was temporarily activated to slowly circulate nutrients and replace the volume of media within the four channels.

Live-cell and immunofluorescence imaging

Imaging was performed using a Nikon TE-2000U inverted microscope controlled by NIS Elements software (Nikon, Japan). Phase-contrast images were captured every 20 min at three locations in each 50 mm channel: at the center and 10 mm from each end (Fig. 1d). Before each time lapse image the locations were defined using NIS Elements software. The first location is set such that it is 10 mm from the inlet of the channel and directly in the center of the flow, roughly 2 mm from the side walls to avoid edge effects (Fig. 1d). Subsequent locations are spaced 10 mm from each other, resulting in three imaging locations spaced equally over the length of the channel in the laminar flow region. Images were obtained using a 10× Nikon Plan Fluor objective. Each image was 1.5 mm × 1.2 mm and contained 1000–2000 cells. Autofocus adjustment using NIS-Elements is performed before each image capture to account for any z-drift.

Monolayers of endothelial cells within the device were prepared for immunofluorescence staining immediately following the flow experiment by washing with warm PBS with Ca^{2+} and Mg^{2+} and fixing in 4% formaldehyde (Fisher Scientific) in PBS. Cells were subsequently washed with PBS and permeabilized with 0.1% Triton-X 100 (Sigma Aldrich). Samples were blocked using 10% goat serum in PBS, and incubated with anti-zonula occluden-1 (ZO-1) antibody (rabbit monoclonal 1:200, Invitrogen) for 1 h at room temperature, washed, and incubated with a goat anti-rabbit secondary antibody (1:200, Alexa Fluor 568, Invitrogen). Samples were subsequently stained for F-actin using