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Accelerated endothelial wound healing on microstructured substrates under flow

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ABSTRACT

Understanding and accelerating the mechanisms of endothelial wound healing is of fundamental interest for biotechnology and of significant medical utility in repairing pathologic changes to the vasculature induced by invasive medical interventions. We report the fundamental mechanisms that determine the influence of substrate topography and flow on the efficiency of endothelial regeneration. We exposed endothelial monolayers, grown on topographically engineered substrates (gratings), to controlled levels of flow-induced shear stress. The wound healing dynamics were recorded and analyzed in various configurations, defined by the relative orientation of an inflicted wound, the topography and the flow direction. Under flow perpendicular to the wound, the speed of endothelial regeneration was significantly increased on substrates with gratings oriented in the direction of the flow when compared to flat substrates. This behavior is linked to the dynamic state of cell-to-cell adhesions in the monolayer. In particular, interactions with the substrate topography counteract Vascular Endothelial Cadherin phosphorylation induced by the flow and the wounding. This effect contributes to modulating the mechanical connection between migrating cells to an optimal level, increasing their coordination and resulting in coherent cell motility and preservation of the monolayer integrity, thus accelerating wound healing. We further demonstrate that the reduction of vascular endothelial cadherin phosphorylation, through specific inhibition of Src activity, enhances endothelial wound healing in flows over flat substrates.

1. Introduction

The vascular endothelium regulates the transport of substances from the blood into the vessel wall [1,2]. Disturbance of this regulatory function, resulting for example from endothelial injury, can cause cardiovascular disease [3]. Understanding and expediting the healing of the endothelium after wounding is thus necessary to avoid focal disease onset.

Cell-to-cell adhesion within a monolayer of endothelial cells (ECs) is ensured by tight junctions and adherens junctions. These are specialized biological structures through which the cohesion of the endothelium is maintained [4]. However, a differentiated endothelium is far from a static sheet of connected cells: Constitutive planar cell movements effect a continuous and dynamic remodeling of the cell junctions [2,5,6].

The migratory potential of ECs and their mechanical interconnection are fundamental factors in the response of a monolayer when its continuity is compromised by a wound [5,6]. ECs react to the open space by polarizing and migrating into the wound area collectively, i.e. under maintenance of cell-to-cell adhesions [7], to reestablish a confluent monolayer [5,6,8–12]. This process relies on a modular control of a number of cellular activities to (i) induce directed migration of the cells at the wound interface (border cells), (ii) modulate the autonomous random migration of the inner cells, and (iii) coordinate cell motion within the endothelium [5,6]. Eventually, this response orients sheet migration toward the wound while preserving monolayer connectivity [6].

The current knowledge on wound healing derives from *in vitro* experiments with monolayers moving on flat substrates [6,9,12]. Yet *in vivo*, ECs interact with a topographically complex basal matrix [13]. The effect of topographical features with size and orientation similar to those presented by the extracellular matrix

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has been analyzed previously using substrates engineered by means of nanoimprint lithography [14,15]. These studies demonstrated that surface texture affects migration and polarization of individual ECs substantially [16,17]. Contact guidance is responsible for these effects. This process is based on the physical confinement of transmembrane receptors of the integrin family, which controls biological contact between the cell and its substrate through the establishment and maturation of focal adhesions [15,18]. Less attention has been paid so far to the influence of topography on entire monolayers as opposed to individual cells [19].

Adherens Junctions (AJs) play a pivotal role in the collective migration of ECs [7,20]. Vascular Endothelial Cadherin (VEC), a member of the cadherin transmembrane protein family localized at AJs of ECs, is necessary for the mechanical cohesion of cells in the monolayer. When VEC is down-regulated *in vitro*, border cells still enter the wound, but lose connection to the inner cell lines [6]. Conversely, when cell-to-cell adhesion is strongly reinforced, directed migration of border cells is hampered and wound closure slows down [5]. Fine modulation of the mechanical linkage between ECs within the range of the above mentioned two extremes appears, therefore, to be critical in facilitating migration toward the wound while maintaining monolayer integrity [7,12]. Factors that influence the stability of AJs are thus likely to affect endothelial wound healing [7]. Among these, flow-mediated endothelial Wall Shear Stress (WSS) and substrate topography are known to play a critical role in the control of vascular permeability *in vivo*. Previous studies reported on the combined effect of substrate topography and flow either on single cell migration or on the morphology of confluent monolayers [21,22]. However, their combined contribution to wound healing, although likely to be important, has never been addressed.

We recently demonstrated that the dynamic state of AJs in the endothelium is regulated through the phosphorylation of VEC [23]. WSS values up to 1.4 Pa activate junctional Src, both *in vivo* and *in vitro*, thus rendering VEC responsive to a number of inflammatory stimuli. In particular, tyrosine phosphorylated-VEC (pY-VEC) can be quickly withdrawn from AJs to induce a transient reduction of the adhesion strength, which increases vascular permeability [23].

Here we investigate the interplay between substrate topography and flow-mediated WSS during wound healing of entire endothelial monolayers. Endothelial monolayers were grown on topographically modified substrates and exposed to controlled levels of WSS within a custom designed parallel plate flow chamber. We recorded and analyzed the wound healing dynamics under variable configurations defined by the relative orientations of the wound, topography, and flow.

2. Material and methods

2.1. Substrate fabrication

Gratings with depth, line width, and pitch of 1 μm were imprinted on 180 μm thick untreated cyclic olefin copolymer (COC) foils (Ibidi, Germany) using nanoimprint lithography (NIL) as previously reported [15,16]. At the end of the fabrication procedure, the substrates were treated with oxygen plasma (100 W for 30 s) to increase the hydrophilicity of the surface and to promote cell adhesion.

2.2. Antibodies

The following primary antibodies were used: Mouse anti-vinculin (V4505) purchased from Sigma Aldrich (USA), goat anti-VEC (Vascular Endothelial Cadherin; #6458) from Santa Cruz Biotechnology Inc. (USA) and rabbit pY658-VEC [23]. The secondary antibodies applied were: goat anti-rabbit HRP (#65-6120), donkey anti-goat-alexa-488 (A11055) and donkey anti-mouse-alexa-488 (A21202), all from Invitrogen (USA).

2.3. Cell culture

Human umbilical vein endothelial cells (HUVEC; Invitrogen, USA) were grown in medium 200PRF supplemented with fetal bovine serum 2% v/v, hydrocortisone 1 μg/ml, human epidermal growth factor 10 ng/ml, basic fibroblast growth factor 3 ng/ml and heparin 10 μg/ml (all reagents from Invitrogen) and were maintained at 37 °C and 5% CO_2. All reported experiments were performed using cells with less than seven passages *in vitro*. The substrates were sterilized by overnight treatment with ethanol and rinsed three times with PBS before starting the coating procedure. The substrates were then coated with gelatin according to the protocol by Lampugnani et al. [24]. The substrates were stored at 4 °C until the seeding of the cells. To generate a confluent monolayer, cells were seeded on COC substrates at high density ($3.5–5 \times 10^4$ cell/cm^2) and cultured for three days.

2.4. Wound healing experiments

A custom designed parallel plate flow chamber was used to apply a constant shear stress to the monolayers during wound healing (Fig. 1). The shear stress applied on the cells (τ) can be expressed as function of the channel dimensions (width, w and height, h), medium properties (viscosity, μ) and volumetric flow rate (Q) using the calculation for wall shear stress in a rectangular channel: $\tau = 6Q\,\mu/wh^2$ [23]. While channel dimensions and medium properties were fixed in our experimental setup (w = 20 mm, h = 0.3 mm, $\mu = 8.4{*}10^{-4}$ Pa*s), the flow rate was controlled using a peristaltic roller pump (Model 66, Harvard Apparatus) to apply WSS of 1.4 Pa to the endothelial cell monolayer. A compliance element was inserted between the roller pump and the flow chamber to dampen flow pulsation.

Before starting the wound healing experiments, cells were labeled using a DiD Vybrant solution (Invitrogen). The cell monolayer was incubated for 40 min at 37 °C with 1 ml of normal growth medium supplemented with 3 μl of the labeling dye. The staining solution was then substituted with fresh growth medium and the samples were kept at 37 °C and 5% CO_2 for 15 min before wounding. A longitudinal wound was mechanically implemented with a pipette tip as described in [25]. Scanning electron microscopy images were acquired to demonstrate that the substrate is not damaged upon wounding the endothelial monolayer (Supplementary Fig. 1). Depending on the dimension of the pipette tip, small (200 μm< width<350 μm) or large (500 μm <width<700 μm) wounds were reproducibly generated. In particular, wounds were always oriented perpendicular to the direction of gratings. After wounding of the monolayer, the substrate was placed in the flow chamber and a constant WSS of 1.4 Pa was applied to the cells.

For the Src inhibition experiments, the specific inhibitor PP1 (Enzo Life Sciences Inc., USA) was added to the culture media at a final concentration of 10 μM as reported in [23]. The monolayer was then incubated at 37 °C and 5% CO_2 for 30 min and afterward the wound was mechanically implemented before placing the substrate into the flow chamber. The medium flowing in the flow chamber was also supplemented with PP1 at the same concentration to sustain the Src inhibition during the entire wound healing experiment.

2.5. Immunostaining

HUVECs were fixed and permeabilized for 3 min with 3% paraformaldehyde (PFA) and 0.5% Triton-X100 in PBS at room temperature (RT). The cells were then post-fixed with 3% PFA in PBS for 15 min. After washing the samples three times for 5 min with PBS, they were incubated with 5% bovine serum albumin (BSA) and 5% donkey serum in PBS for 1 h at RT. The samples were incubated either with anti-vinculin primary antibody together with TRITC-phalloidin (Sigma, USA) or with anti-VEC together with anti-pY658-VEC primary antibodies overnight at 4 °C. Subsequently, the samples were rinsed four times for 1 h with 5% BSA in PBS and then were incubated with anti-mouse-alexa-488 secondary antibody and goat anti-rabbit HRP together with anti-goat-alexa-488 secondary antibodies for 45 min at RT. Finally, the samples were washed three times (1 h each) in PBS, post-fixed for 2 min in 3% PFA, briefly washed again with PBS, mounted with DAPI-containing Vectashield (Vector Labs Inc., USA) and immediately imaged.

2.6. Video acquisition

Live wound healing was imaged using an inverted Nikon-Ti wide-field microscope (Nikon, Japan) equipped with an Orca R-2 CCD camera (Hamamatsu Photonics, Japan) and an incubated chamber (Life Imaging Services, Switzerland). Both the flow chamber and the medium reservoir were maintained at a controlled temperature of 37 °C and CO_2 concentration of 5%. Images were collected with a 20X, 0.45 NA long-distance objective (Plan Fluor, Nikon, Japan). The experiment was started to automatically collect images in the TRITC channel with a time resolution of 15 min for a total of 16 h. Using the large image function (NIS Elements, Nikon, Japan), a field of 3 by 3 single images was acquired for each set position. Focal drift during the experiments was eliminated using the scope's PFS autofocus system. At the end of the experiment, the resulting time-lapses of each set position were converted into individual 16 bit movies for analysis.