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Facile endothelium protection from TNF-α inflammatory insult with surface topography

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ABSTRACT

Adverse events triggered by the direct contact between blood and synthetic materials constitute a sincere shortcoming of cardiovascular implant technology. A well-connected autologous endothelium, generated through the process of endothelialization, impedes such interaction and endows the implant luminal interface with optimal protection. The endothelialization of artificial substrates is the result of a complex interplay between endothelial cells (ECs), surface topography, and flow-generated wall shear stress (WSS). This is however tainted by the pro-inflammatory signaling, typical of cardiovascular patients, which compromises endothelial integrity and survival. Here, we challenge human endothelial monolayers with the pro-inflammatory factor TNF-α under realistic WSS conditions. In these experimental settings we demonstrate that the simple contact between ECs and an optimized surface geometry can inhibit NF-kB activation downstream of TNF-α yielding increased stability of VE-Cadherin mediated cell-to-cell junctions and of focal adhesions. Therefore the here-presented topographic modification can be implemented on a range of artificial substrates enabling their endothelialization under supra-physiological flow and in the presence of pro-inflammatory insults. These new findings constitute an important step toward achieving the full hemocompatibility of cardiovascular implants.

1. Introduction

The absence of a fully hemocompatible blood-foreign surface interface represents a serious shortcoming of cardiovascular implant technology. An optimal protection against the adverse events triggered by the contact between blood and synthetic materials can be produced by an autologous endothelial cell (ECs) layer through the process of surface endothelialization [1]. Intense research efforts have therefore focused on strategies to support EC adhesion, proliferation, differentiation, and migration on biomaterials [2]. These included the chemical, physical, or biological functionalization of the surface, or the generation of intervening layers with favourable properties [3]. Clinical tests of these endothelialization strategies were performed on coronary stents [4] and vascular grafts [5] yielding variable, but altogether unsatisfying, outcomes [5]. To date, long-term endothelialization of foreign body surfaces remains an unmet need of cardiovascular implant technology.

Endothelialization strategies have been typically tested and validated in simplified *in vitro* or *in vivo* model systems [5] which failed to reproduce fundamental clinical aspects such as the chronic inflammatory environment present in most cardiovascular patients and the hemodynamic conditions generated at the luminal interface upon device deployment [6–9]. Locally, these factors can overlap to produce a hostile environment for EC survival [10,11]. Thus, successful endothelialization approaches must be developed in experimental settings able to reproduce both the pro-inflammatory signaling and the actual hemodynamic environment to which ECs will be exposed to in patients [12].

Chronic endothelial inflammation is a common co-morbidity in cardiovascular patients [6–8]. Over a period of several years this condition sustains a decay of tissue homeostasis and mass

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transport control, the hallmarks of a healthy endothelium [7]. Eventually, it leads to the loss of endothelial function and the concomitant activation of inflammatory signaling at the origin of coronary artery disease (CAD) and of other cardiovascular pathologies [6,7,10,13]. In particular, the activation of ECs is mediated by circulating pro-inflammatory cytokines (TNF-α and IL-2, IL-6, and IL-8) [7,14–16] and induces the expression of clotting or coagulation factors (e.g. von Willebrand Factor, thromboxane, prostacyclin), fibrinolysis factors, cell adhesion molecules (VCAM-1), and intercellular adhesion molecules (ICAM-1) [11,17] which in turn lead to the loss of tissue integrity and denudation of the substrate [7,11,18]. A master regulator of gene expression patterns orchestrating endothelial inflammation is the transcription factor NF-kB. Its translocation to the cell nucleus is triggered by circulating TNF-α and enables the onset of the inflammatory response in ECs [14,19].

The local hemodynamic conditions deeply impinge on the inflammatory signaling cascade either contributing to define foci of inflammation [7] or to create protective environments where endothelial integrity is preserved despite the pro-inflammatory milieu [14]. The level of flow-mediated wall shear stress (WSS) directly modulates the dynamic stability of cell-to-cell junctions in endothelia experimentally exposed to pro-inflammatory insults, both *in vitro* and *in vivo* [20–23]. This process is mediated by the phosphorylation of Vascular Endothelial Cadherin (VEC), a junctional protein mediating homotypic contacts at the adherens junctions (AJs) and therefore essentially contributing to monolayer integrity [20]. Similarly, WSS affects the stability of integrin-mediated focal adhesions (FAs) to the substrate [24]. Here, the adaptor protein Vinculin mediates the interaction between the adhesion plaque and the actin cytoskeleton enabling FA maturation [25]. Importantly, Vinculin is also involved in the stabilization of AJs therefore assuming a pivotal role in the maintenance of monolayer integrity [21,26].

A promising approach to foster endothelialization of artificial materials is represented by the nano and microscale structuring of luminal interfaces with rationally-designed surface geometries [27]. We previously demonstrated that anisotropic topographies (alternating lines of ridges and groves) with horizontal and vertical feature size in the micron range are able to interfere with the machineries mediating EC activities under flow [24,28,29]. In this case, the effect of topography is exerted through the physical modulation of adaptor protein recruitment and FA maturation [24,28]. In particular, FAs established by ECs along gratings oriented parallel to flow support directional migration [29] and contribute to reinforce AJs by inhibiting VEC phosphorylation [20,29]. Altogether, endothelial monolayers are reinforced by the contact with oriented anisotropic topographies and are thus able to preserve their integrity well beyond physiological WSS values [30]. The attainment of these enhanced properties paves the way to new endothelialization strategies applicable to active cardiovascular devices which function at supraphysiological hemodynamic conditions, such as ventricular assist devices (VADs, [12,31]). However, the available information on the performance of microstructured substrates upon endothelialization, under combined effect of flow and pro-inflammatory signals is incomplete. While some indications of a protective effect mediated by aligned nano fibrillar substrates [32] and micrograting [33] were obtained for ECs under static conditions, dynamic settings were only explored in the absence of circulating cytokines [34] or in subconfluent conditions [35].

Here we report, novel findings on the effect of oriented topographies in preserving endothelial integrity upon pro-inflammatory insults. The endothelialization of synthetic substrates is challenged through a combination of pharmacological treatment with circulating TNF-α and flow-generated physiological or supraphysiological WSS values, altogether mimicking the environment expected at the luminal surface of cardiovascular devices. The resulting protective effect is measured by monitoring the nuclear translocation and functional activity of NF-kB and the integrity of monolayer junctions and substrate adhesions in comparison with identical, flat substrates. Our results demonstrate a strong protective and anti-inflammatory effect of gratings upon substrate endothelialization under a range of physiological and supraphysiological flow conditions.

2. Materials and methods

2.1. Substrate fabrication

Gratings with depth, ridge width and pitch of 1 μm were imprinted on 180 μm thick cyclic olefin copolymer (COC) foils (Ibidi, Germany) using nano-imprint lithography (NIL) [28]. Before usage, the substrates were treated with plasma (100 W for 30 s, 1 ± 0.2 mbar) to increase hydrophilicity and were coated with gelatin to promote cell adhesion. The elastomeric substrates (Supplementary Fig. 1) were made of polydimethylsiloxane (PDMS, Dow Corning, USA) at a 1:10 mixing ratio and fabricated through standard soft lithography. In brief, the mixed PDMS was degassed in a vacuum chamber and afterwards immediately poured onto untreated COC substrates containing gratings. After pouring on the COC molds, the PDMS was briefly degassed for a second time and cured for 4 h at 60 °C. The cured PDMS patches were then carefully separated from the COC mold. CTRL patches were created similarly by pouring PDMS on flat COC substrates.

2.2. Cell culture and treatments

Primary human umbilical vein endothelial cells (HUVECs; Invitrogen, USA) were grown in medium 200PRF supplemented with fetal bovine serum 2% v/v, hydrocortisone 1 mg/ml, human epidermal factor 10 ng/ml, basic fibroblast growth factor 3 ng/ml and heparin 10 mg/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 surfaces were sterilized with 70% ethanol and rinsed three times with PBS before starting the coating procedure. They were subsequently coated with 1,5% gelatin (104070, Merck Millipore, USA) [29–31] and stored at 4 °C until the seeding of the cells. Importantly, the gelatin coating procedure applied to the substrates does not alter the surface topography [36] as confirmed by the SEM images of gratings after coating, which confirm the retention of topographic feature size and periodicity (Supplementary Fig. 2). To generate a confluent monolayer, cells were seeded on COC substrates at high density (3.5–5 x 10^4 cell/cm^2) and cultured for three days. The adhesion of endothelial cells cultured on gratings was confined to the top of the ridges. Deep grooves are not accessible to the cell membrane, which forms arcs bridging on top of them [28]. This specific conformation of the cell layer can be appreciated in the SEM reported in Supplementary Fig. 2.

For the measurement of endothelial activation upon treatment with pro-inflammatory cytokines, endothelial monolayers grown on flat substrates or gratings were treated with TNF-α at various concentrations for 18 h before immunostaining assays (Supplementary Fig. 3). To inhibit myosin II, HUVEC monolayers were treated with 50 μM blebbistatin for 45 min.

2.3. Experiments under flow

A custom-designed parallel plate flow chamber was used to apply a constant shear stress to endothelial cell monolayers