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Spatial patterning of endothelium modulates cell morphology, adhesiveness and transcriptional signature

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ABSTRACT

Microscale and nanoscale structures can spatially pattern endothelial cells (ECs) into parallel-aligned organization, mimicking their cellular alignment in blood vessels exposed to laminar shear stress. However, the effects of spatial patterning on the function and global transcriptome of ECs are incompletely characterized. We used both parallel-aligned micropatterned and nanopatterned biomaterials to evaluate the effects of spatial patterning on the phenotype of ECs, based on gene expression profiling, functional characterization of monocyte adhesion, and quantification of cellular morphology. We demonstrate that both micropatterned and aligned nanofibrillar biomaterials could effectively guide EC organization along the direction of the micropatterned channels or nanofibrils, respectively. The ability of ECs to sense spatial patterning cues were abrogated in the presence of cytoskeletal disruption agents. Moreover, both micropatterned and aligned nanofibrillar substrates promoted an athero-resistant EC phenotype by reducing endothelial adhesiveness for monocytes and platelets, as well as by down-regulating the expression of adhesion proteins and chemokines. We further found that micropatterned ECs have a transcriptional signature that is unique from non-patterned ECs, as well as from ECs aligned by shear stress. These findings highlight the importance of spatial patterning cues in guiding EC organization and function, which may have clinical relevance in the development of vascular grafts that promote patency.

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1. Introduction

Cardiovascular disease is the major cause of morbidity and mortality in the US [1]. In particular, atherosclerotic diseases can lead to plaque rupture, thrombosis and/or embolism, causing myocardial infarction and stroke. Atherosclerosis is a chronic inflammatory disease that begins with endothelial activation [2,3]. Upon exposure to hypercholesterolemia or other conditions that cause atherosclerosis, endothelial cells (ECs) begin to express adhesion molecules and chemokines. Vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1) are adhesion proteins that facilitate the adhesion of leukocytes to the arterial wall [4,5] and eventually contribute to the accumulation of atherosclerotic plaque. Monocyte chemoattractant protein-1 (MCP1) is a chemokine that is associated with the formation of atherosclerotic lesions [6].

The spatial distribution of atherosclerotic lesions strongly correlates with endothelial morphology. ECs aligned along a straight segment of an artery appear to be resistant to vascular inflammation, whereas the non-aligned endothelial cells at a branch or bifurcation express biological properties that promote atherosclerosis [7]. The expression level of the pro-atherosclerotic molecules and the location of vascular lesions are often found in the sites of the vessel where the geometry is branched or curved [2]. At bends, branches and bifurcations, disturbed flow predominates, and the ECs are not aligned. We speculate that the biology of aligned ECs, possibly mediated by integrin–extracellular matrix (ECM) signaling, might be atheroprotective.

Interactions between the cell and the ECM are critical to cell phenotype and function, and should be considered in the design of

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medical devices [8]. Grooves, ridges, steps, pores, wells, and nodes patterned into substrates modify cellular response in fibroblasts, neurons, epithelial cells, macrophages, endothelial cells, and smooth muscle cells [9–19]. Micropatterning influences both the morphology and migration of ECs [20–26]. However, prior studies of the effect of patterned substrates on EC morphology have provided only limited information about function, and little is known about effects on EC adhesiveness, a key property regulating inflammation and thrombosis [2]. In addition, these initial studies have been limited to patterned topography at the micron length scale, due to resolution limitations of the patterning techniques. Nanoscale features can be more biologically relevant to investigate than topography in the micron range since the cells interact with many features (pores, fibers, ridges) of the native ECM, which is in the nanometer scale [8]. A thorough understanding of cell–matrix interactions, specifically at the nanoscale, would significantly contribute to the design of biomaterials that provide inductive signaling cues to ECs.

We hypothesized that matrix-mediated parallel alignment of ECs may promote an athero-protective phenotype by modulating cell morphology, adhesiveness, and transcriptome. To test this hypothesis, we used a micropatterned polydimethylsiloxane (PDMS) substrate to evaluate the effects of parallel-aligned microchannels on the phenotype and function of ECs, based on gene expression profiling of adhesion molecules, functional characterization of monocyte and platelet adhesion, and quantification of cellular alignment. We further extended this work to investigate the phenotype of ECs on oriented nanofibrillar collagen, which is a naturally-derived and biodegradable ECM.

2. Materials and methods

2.1. Micropatterned substrates

Polydimethylsiloxane (PDMS) microgrooves were fabricated using soft lithography techniques [27] with some modifications. A 10-μm thick layer of a negative photoresist (SU-8 3010, Microchem) was spin-coated onto silicon wafers (mechanical grade, University Wafer) for 30 s at 3500 rpm and UV-exposed for 8.5 s at 20 mW/cm^2 (OAI, light source centered at 365 nm) through a transparency mask (FineLine Imaging) and long pass UV filter (PL-360-LP Omega Optical). The AutoCAD generated transparency mask contained microgroove patterns of 30 μm width and spacing. Previous to UV exposure, SU-8 was baked on a hotplate (ramp from 65 to 95 °C and held for 6.5 min). Before development, SU-8 was submitted to post exposure baking (ramp from 65 to 95 °C and held for 2.5 min). After development of cured SU-8 features for 4.5 min in liquid with mild agitation (SU-8 Developer), N_2 gas dried wafers were silanized in a trimethylchlorosilane (Aldrich) atmosphere for 3 h. PDMS (Sylgard 184, Dow Corning) prepolymer and curing agent were mixed 10:1 (Thinky), cast on the SU-8 microstructures, cured for 24 h at 70 °C, and then peeled from the SU-8 master.

To facilitate mold release, the cured PDMS-184 micropattern molds were O_2 plasma treated with 66.6 Pa and 80 W in an oxygen plasma asher (Branson/IPC-S3003) for 15 s, and then silanized as above described for 24 h. PDMS-based micropatterned cell-culture substrates were fabricated by casting mixed 10:1 PDMS-182 (Sylgard 182, Dow Corning) [28] and degassed before curing at 70 °C for 24 h. The obtained micropatterned PDMS substrates had microchannel dimensions of 30 μm width, 30 μm spacing and 10 μm in depth (Fig. S1). The micropatterned substrates were plasma treated as above immediately before incubating with rat tail collagen type I (0.35 mg/mL, BD Biosciences) before seeding cells.

2.2. Fabrication of aligned collagen strips

Strips of anisotropic nanofibrillar collagen matrices were produced by a syringe needle extrusion-based fibrillogenesis method, modified from Lai et al. [29]. Briefly, high concentration rat tail collagen I was dialyzed to 30 mg/mL using polyethylene glycol (Fluka) at 4 °C using semi-permeable cellulose dialysis tubing (Fisher). To create anisotropic collagen, monomeric collagen was extruded in the form of a strip at a velocity 3.2 mL/min from a syringe into a 10× phosphate buffered saline (PBS), pH9 PBS buffer at 37 °C to induce fibrillogenesis along the direction of extrusion. The anisotropic collagen strips were then manually deposited onto a glass slide. Strips of non-aligned collagen fibrils, or the randomly oriented control, were produced by extruding at a slow rate of 0.02 mL/min to produce strips of random collagen, with similar thickness and width as the aligned collagen strips. The collagen then completely dried under a laminar flow hood for 12 h and was then rinsed several times with MilliQ water.

2.3. Scanning electron microscopy (SEM)

Samples were processed for routine SEM, according to previous publications, using a Hitachi S-3400N VP scanning electron microscope [30].

2.4. In vitro culture experiments

Primary human dermal microvascular endothelial cells (ECs) were grown in EGM-2 MV (Lonza) media, supplemented with EGM-2 MV SingleQuot kit (Lonza), at 37 °C and 5% CO_2. The micropatterned PDMS and collagen substrates were sterilized in 70% ethanol for 10 min and then washed with PBS before cell seeding. ECs were plated at a seeding density of 23,000–28,000 cells/cm^2 and incubated for 24 h on the substrates. The ECs were used below passage 14.

Where specified, cytoskeletal inhibitors were used to evaluate the role of actin or microtubules on cellular elongation and alignment. Immediately after plating the ECs on the collagen substrates, the ECs were incubated for 24 h with cytoskeletal inhibitors cytochalasin D (100 nM, Sigma) or nocodazole (1 μM, Sigma), which disrupt the actin and microtubule networks, respectively ($n = 3$).

2.5. Immunofluorescence staining

At indicated time points, samples were fixed in 4% paraformaldehyde (Alfa Aesar) and immunofluorescently stained for antibodies against Ki67 (Dako Cytomation), paxillin (BD Transduction Labs), focal adhesion kinase (FAK, BD Transduction Labs), and β-tubulin (Sigma), based on previous protocols [29,31]. In addition, phalloidin (Invitrogen) and Hoechst33342 dye (Invitrogen) were used to visualize the F-actin and nuclei, respectively.

2.6. Analysis of cellular alignment

Cell alignment and shape were analyzed using ImageJ to conduct measurements, as previously described ($n = 3$ micropatterned substrates; $n = 6$ collagen nanofibrillar substrates) [29]. The angle of orientation was defined as the angle formed by the cell's principal axis, relative to the direction of the substrate (i.e. microchannels or nanofibrillar collagen). A minimum angle of 0° denoted parallel alignment from the substrate's axis and a maximum of 90° suggested perpendicular alignment. For quantification of cells on the random control, an arbitrary axis was selected.

We further validated the cell alignment analysis by quantifying the order parameter (S), where $S = <(3\cos^2\theta - 1)/2>$, and θ is the angle of alignment ($n = 3$ microchannels; $n = 6$ nanofibrillar collagen) [29]. A perfectly aligned distribution where θ is 0°, relative to the substrate axis, would indicate $S = 1$. The cell shape index (CSI) provided an indicator of cell morphology by quantifying the extent of elongation in the cellular shape, where CSI = (4π*Area)/(Perimeter^2) [19,29]. A CSI value of 1 approximates the shape of a circle and a value of 0 depicts that of a straight line.

2.7. Monocyte adhesion and platelet adhesion assays

Confluent ECs on the PDMS or collagen substrates were stimulated with tumor necrosis factor-α (TNF-α, Sigma) at 2.5 ng/mL for 7 h. Human monocytes (U937, ATCC) were fluorescently labeled with Cell Tracker Red and then allowed to adhere onto the substrates for 45 min with gentle rocking to promote even cell distribution. After 45 min, the unbound monocytes were washed away by rinsing twice with PBS before quantifying the number of bound monocytes. Cells cultured on aligned or random substrates were imaged in five representative fields, and the remaining monocytes in each image were quantified ($n \geq 3$). The level of monocyte adhesion was expressed as a relative fold change relative to the control, non-patterned substrate or randomly aligned collagen.

Similar experiments were performed using primary human apheresis platelets (Stanford Blood Center) to compare platelet adhesion onto ECs cultured on aligned or randomly oriented nanofibrillar collagen. The platelets were exposed to TNF-treated ECs on either aligned or randomly oriented collagen. After 30 min, the samples were fixed in paraformaldehyde and immunofluorescently stained for platelet marker CD41 (Abcam). Analysis was performed similar to the monocyte adhesion assay, taking images in five representative fields ($n = 6$). The bound platelets were quantified and expressed as a relative fold change with respect to platelets on randomly aligned collagen.

2.8. Gene expression analysis

The ECs on the micropatterned or control PDMS substrates were lysed with Trizol (Invitrogen), followed by RNA isolation using the RNEasy kit (Qiagen). For DNA microarray analysis, RNA quality was verified by Agilent Bioanalyzer, and then the cDNA was synthesized and labeling according based on previous publications [32]. The samples were hybridized to Affymetrix Human Gene 1.0 ST GeneChips and then scanned on an Affymetrix 3000 scanner according to standard protocols ($n = 2$). Fluorescence intensities were normalized according to the manufacturer's instructions and are available in the Gene Expression Omnibus (GEO) database (accession no. GSE42596).

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