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The influence of electrospun scaffold topography on endothelial cell morphology, alignment, and adhesion in response to fluid flow

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Abstract

Bioengineered vascular grafts provide a promising alternative to autografts for replacing diseased or damaged arteries, but necessitate scaffold designs capable of supporting a confluent endothelium that resists endothelial cell (EC) detachment under fluid flow. To this end, we investigated whether tuning **electrospun** topography (i.e. fiber diameter and orientation) could impact EC morphology, alignment, and structural protein organization with the goal of forming a confluent and well-adhered endothelium under fluid flow. To test this, a composite polymer blend of Poly(ϵ -caprolactone) (PCL) and type I collagen was electrospun to form scaffolds with controlled fiber diameters ranging from approximately 100 nm to 1200 nm and with varying degrees of fiber alignment. ECs were seeded onto scaffolds, and cell morphology and degree of alignment were quantified using image analysis of fluorescently stained cells. Our results show that ECs form confluent monolayers on **electrospun** scaffolds, with cell alignment systematically increasing with a larger degree of fiber orientation. Additionally, cells on aligned **electrospun** scaffolds display thick F-actin bundles parallel to the direction of fiber alignment and strong VE-cadherin expression at cell-cell junctions. Under fluid flow, ECs on highly aligned scaffolds had greater resistance to detachment compared to cells cultured on randomly oriented and semi-aligned scaffolds. These results indicate that scaffolds with aligned topographies may be useful in forming a confluent endothelium with enhanced EC adhesion for vascular tissue engineering applications.

1. Introduction

Currently, there are more than one million vascular procedures performed each year in the US to treat various forms of cardiovascular disease such as coronary and peripheral artery disease [Nieponice et al., 2008]. Many of these procedures include bypass surgery, wherein autologous vessels are used to reroute blood flow around occluded or diseased arteries.

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Author Disclosure Statement

No conflicting interests exist

While autologous vessels are considered the gold standard for bypass grafts, this procedure can result in donor site morbidity or is often not feasible since many patients lack a suitable vein or artery for bypass grafting [Cameron et al., 1996, Conte, 1998]. As a result, there has been significant interest in using bioengineered vascular grafts fabricated from both synthetic and natural sources as an alternative to autologous grafts.

A critical design requirement for small-diameter (<6 mm) bioengineered vascular grafts is the formation of a continuous monolayer of endothelial cells (ECs) on the lumen of the construct. This is typically accomplished by seeding autologous ECs, such as venous ECs [Grenier et al., 2003, L'Heureux et al., 2007] or endothelial progenitor cells [Kaushal et al., 2001, Sieminski et al., 2005] on the lumen surface of the vascular graft prior to implantation. In native vessels, ECs play an important role in regulation of vascular tone, tissue homeostasis, and regulation of nutrient transport across the vessel wall [Shireman et al., 1996]. In addition, the endothelium forms a selectively permeable, antithrombogenic barrier between the circulating blood and vessel wall [Cines et al., 1998]. Without a durable and adherent endothelium, vascular grafts are susceptible to failure after bypass surgery as a result of neointimal hyperplasia and thrombosis, typically caused by a lack of endothelial coverage on the graft [L'Heureux et al., 2006, Seifalian et al., 2002, Williamson et al., 2006]. Therefore, an ideal bioengineered vascular graft should possess a continuous monolayer of ECs that functions similar to the native endothelium while remaining adherent under physiological flow conditions.

It is well known that the morphology and cytoskeleton arrangement of ECs in native arteries directly affects their ability to function and resist detachment under physiological fluid flow [Langille et al., 1991]. The endothelium in native vessels is composed of ECs that are aligned with the direction of blood flow in straight vessel segments. These morphological properties (i.e. shape and orientation) are directed by flow-mediated mechanotransduction, where mechanical forces are converted into cellular activity through cell-surface membrane protein mechanoreceptors and mechanosensitive ion channels, among others [Davies, 1995]. Under flow conditions, mechanotransduction induces cytoskeletal rearrangement, specifically F-actin, in ECs exposed to fluid flow [Davies, 1995, Langille et al., 1991]. ECs located at sites of disturbed fluid flow *in vivo*, i.e. at vessel branches or atherosclerotic lesions, or under static conditions *in vitro*, display a polygonal morphology where F-actin is organized at the periphery of the cells. Upon exposure to high shear stress, cells align with the direction of flow and F-actin rearranges to create thick bundles of stress fibers parallel to the direction of fluid flow. Furthermore, the reorientation and alignment of ECs, in addition to F-actin organization, substantially increases the cells ability to resist deformation and detachment under shear stress [Davies, 1995, Langille et al., 1991, Mott et al., 2007]. Therefore, in this study, we hypothesized that if ECs can be directed to adopt an *in vivo*-like morphology, alignment, and F-actin organization in the direction of fluid flow, their resistance to detachment may increase as a result.

An effective method that has been employed to achieve an endothelium with *in vivo* like morphology and alignment is *in vitro* fluid flow preconditioning prior to implantation [Baguneid et al., 2004]. This approach utilizes fluid flow bioreactors to expose the lumen of the graft to incremental increases in hydrodynamic shear stress to achieve an aligned and well-adhered endothelium. While effective, this approach is time and resource intensive, typically requiring several days to weeks to achieve a conditioned endothelium which can accept physiologic levels of shear stress without detachment [Niklason et al., 1999, Quint et al., 2011]. Alternatively, a potentially promising method to form an endothelium with *in vivo*-like morphology and alignment may be through the use of a phenomenon called contact guidance, where substratum surface topography can direct cell spreading [Flemming et al., 1999]. Several groups have shown that EC morphology, orientation, and cytoskeleton can be

controlled on a variety of topographic features including nanoscale silicone and polyurethane grooves [Liliensiek et al., 2010, Uttayarat et al., 2010, Uttayarat et al., 2005], micron-diameter methacrylic terpolymer fibers [Heath et al., 2010, Veleva et al., 2009], and nanoscale poly(dimethylsiloxane) waves [Jiang et al., 2002]. Despite these studies, it remains unclear how ECs respond to topographical features such as fiber diameter and orientation, specifically for fiber diameters in the sub-micron range, in an effort to control cell morphology and alignment to increase cell adhesion strength under hydrodynamic shear stress. Therefore, the main objective of the current study is to examine the effects of electrospun Poly(ϵ -caprolactone) (PCL)/type I collagen fiber diameter and orientation on EC morphology, alignment, and structural protein organization with the goal of forming a confluent endothelium that resists detachment under fluid flow. To accomplish this, scaffold topography was tuned by adjusting electrospinning parameters to systematically vary fiber diameter and orientation, concurrently. Cell morphology (cell area, aspect ratio, and length of long axis) and degree of alignment were quantified by performing image analysis of fluorescently stained cells and expression/organization of two principal structural proteins (VE-cadherin and F-actin) and a cell-substrate linker protein (vinculin) was observed by immunofluorescence imaging. EC monolayers on scaffolds were exposed to fluid shear in a parallel plate flow chamber and cell detachment was quantified to elucidate the effect of scaffold diameter and orientation on EC adhesion to the scaffold.

2. Materials and Methods

2.1. Scaffold fabrication

Scaffolds consisting of a polymer blend of type I collagen and PCL were electrospun similar to a method previously reported by Lee et al. [Lee et al., 2008]. Briefly, 1:1 (weight ratio) blends of type I collagen derived from calf skin (Elastin Products Co., Owensville, MO, USA) and PCL (Lactel Absorbable Polymers, Pelham, AL, USA) were prepared in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma Aldrich, St. Louis, MO, USA). PCL/collagen concentrations of 5%, 10%, and 15% (w/v) were used to fabricate scaffolds with varying fiber diameters. To form scaffolds with random fiber orientations, PCL/collagen solutions were electrospun onto a grounded stationary tissue culture polystyrene (TCPS) substrate. To fabricate scaffolds with varying fiber orientation, PCL/collagen solutions were electrospun onto TCPS substrates attached to a grounded rotating aluminum mandrel at 1500 and 3000 rpm (with corresponding linear velocities of 4.0 and 8.0 m/s, respectively). Approximately 1 ml of each solution was used to fabricate PCL/collagen scaffolds with varying fiber diameters and orientations by varying electrospinning parameters (refer to Supplemental Table 1). The scaffolds were then placed in a vacuum desiccator overnight to remove all residual solvent.

2.2. Scaffold characterization

Scaffold morphology was investigated using field emission scanning electron microscopy (SEM) using a LEO 1550 field emission SEM (Carl Zeiss, Thornwood, NY, USA). SEM images were then analyzed using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA) to determine fiber diameter and degree of fiber orientation. A total of 50 random fibers per image were manually measured to determine fiber diameter and angle of orientation ($n = 5$ images/scaffold, $n = 250$ fibers total). The degree of fiber orientation was characterized by the angular standard deviation for a wrapped normal distribution using the method previously described by Basher et al. [Basher et al., 2006].

2.3. Cell culture and cell seeding

Primary human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection (Manassas, VA, USA) and were used to perform cell studies. All

cells were used below passage 5 and cultured in Medium 200PRF supplemented with 2% fetal bovine serum, 1 $\mu\text{g/ml}$ hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 $\mu\text{g/ml}$ heparin (Invitrogen, Carlsbad, CA, USA) in a 37°C, 5% CO₂ incubator. Before cell seeding, scaffolds were sterilized by immersion in 70% ethanol for 1 hr followed by 3 washes in sterile phosphate buffered saline (PBS). HUVECs were then seeded onto the scaffolds at a density of 1.5×10^4 cells/cm².

2.4. Cell morphology and alignment

EC morphology and degree of alignment were assessed 3 days post-seeding by using image analysis of Calcein AM stained cells. This time point was chosen based on preliminary data showing cell confluence at 3 days for the seeding density used (data not shown). Briefly, scaffolds were incubated in medium containing Calcein AM (Molecular Probes, Eugene, OR, USA) at a final concentration of 5 μM for 30 min at 37°C. Scaffolds were washed twice with sterile PBS, culture medium was added to each well, and fluorescent cells were imaged using a Leica DMI6000-B microscope (Leica, Wetzlar, Germany). Images were then analyzed with Image J software (National Institutes of Health, Bethesda, MD) to determine cell morphological properties including projected area, aspect ratio, and length of long axis. The degree of cell alignment was characterized by measuring the angle of cell orientation relative to the vertical axis of the image. Care was taken to ensure that cell-seeded samples were imaged with the direction of mandrel rotation parallel to the vertical axis of the image. A total of at least 10 images ($n = 10$) per scaffold condition were analyzed using this method. The degree of cell orientation was characterized by the angular standard deviation for a wrapped normal distribution as previously described.

2.5. Immunofluorescence staining

Expression and organization of F-actin, VE-cadherin, and vinculin were visualized by immunostaining ECs on scaffolds after 3 days post-seeding. Briefly, all samples were fixed in 3.7% paraformaldehyde (EMD chemicals, Gibbstown, NJ, USA) for 15 min and washed with PBS 3 times for 5 min each. Next, cells were permeabilized by incubation in 0.1% Triton X-100 (Sigma Aldrich) for 5 min, washed 3 times with PBS for 5 min each, then incubated in 1% bovine serum albumin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 30 min to reduce non-specific background staining. For VE-cadherin staining, the samples were incubated for 1 hr in rabbit polyclonal anti-VE-cadherin primary antibody (1:200, Abcam, Cambridge, MA, USA). The samples were then washed with PBS and incubated for 1 hr in goat anti-rabbit secondary antibody (1:100, DyLight 488, Jackson ImmunoResearch, West Grove, PA, USA). To stain for vinculin, samples were incubated for 1 hr in mouse monoclonal anti-vinculin primary antibody (1:100, Abcam), washed with PBS, and incubated for 1 hr in goat anti-mouse secondary antibody (DyLight 594, Jackson ImmunoResearch). For F-actin staining, samples were incubated in rhodamine phalloidin (Invitrogen) for 20 min. All samples were counter-stained using Vectashield with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) to visualize cell nuclei and imaged using a Leica DMI6000-B microscope.

2.6. Cell adhesion assays

ECs on scaffolds were exposed to hydrodynamic shear stress to test cell adherence using a custom parallel plate flow chamber (PPFC) of similar design to that previously described [Kreke et al., 2008]. After 3 days of static culture, EC-seeded scaffolds were mounted in the PPFC and exposed to shear stresses of 20 and 40 dyne/cm² for 1 hr according to the parallel plate approximation as previously described [Kreke et al., 2008]. These shear stresses were chosen because they represent the average (20 dyne/cm²) and maximum (40 dyne/cm²) physiologic shear stress that ECs experience *in vivo* [Silver et al., 2006]. Samples were

mounted in the PPFC such that the direction of fluid flow was parallel to the direction of electrospun fiber alignment (i.e. mandrel rotation). The PPFC was connected to a media reservoir and centrifugal pump (Ismatec BVP-Z, Cole-Parmer, Vernon Hills, IL, USA) to form a closed flow loop. Cell culture media was maintained at 37°C and under a 5% CO₂. Prior to flow, cell nuclei were stained with Hoechst 33342 live cell stain (Invitrogen). The PPFC with EC-seeded scaffolds were then mounted on an imaging stage and imaged at 6 random locations on each scaffold at 0, 5, 10, 20, 30, 40, 50, 60 min after the onset of flow. Three separate flow experiments were conducted for each scaffold condition (n = 3) to yield a total of n = 18 images for each scaffold condition. Image J software was used to count cell nuclei in each image, and the strength of cell adhesion was represented by the percentage of cells remaining after the onset of flow relative to the initial images before flow (0 min).

2.7. Statistical analysis

All values are reported as the mean $\pm$ standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a significance criterion of $p < 0.05$. For assessment of scaffold fiber diameter and alignment, 50 random fibers from n = 5 images per scaffold condition were used (250 fibers total). To determine cell morphology and alignment, a total of at least 10 images (n = 10) per scaffold condition were analyzed. Cell studies were repeated to ensure reproducibility of trends.

3. Results

3.1. Scaffold topography

Electrospinning was used in this study to fabricate fibrous PCL/collagen scaffolds with variable fiber diameters and degrees of alignment. By tuning polymer solution concentration, mandrel rotation rate, and electrospinning parameters (Supplemental Table 1), a 3 x 3 scaffold property matrix was formed. This 3 x 3 scaffold property matrix is displayed in Figure 1, where representative SEM images demonstrate that both fiber diameter and fiber alignment were systematically controlled using the aforementioned fabrication parameters. Quantitative analysis of the SEM images showed that fiber diameters produced from the 5%, 10%, and 15% (w/v) polymer solutions were roughly 100 nm, 300 nm, and 1200 nm, respectively (Figure 2, Table 1), and will be referred to hereafter as “100-nm”, “300-nm”, and “1200-nm”, respectively. Scaffold fiber alignment was controlled by electrospinning onto a stationary TCPS substrate or rotating the TCPS substrate on a mandrel at 1500 and 3000 rpm. Histograms of fiber orientation were plotted for each scaffold condition and show close agreement to the wrapped normal distribution (Supplemental Figure 1). These results indicate that the degree of fiber alignment systematically increased with greater mandrel rotation rates, while the angular standard deviation systematically decreased (Table 1). Scaffolds electrospun at static conditions, 1500 rpm and 3000 rpm will be referred to hereafter as “random”, “semi-aligned”, and “fully-aligned”, respectively, to describe the degree of fiber orientation. Additionally, increasing the mandrel rotation rate, and thus increasing fiber alignment, did not have a statistically significant influence on scaffold fiber diameter (Figure 2).

3.2. Cell morphology and alignment

To assess the effect of scaffold topography on cell morphology and alignment, ECs were seeded onto the scaffolds and visualized using a live-cell stain (Calcein AM). Three days post-seeding, the cells were attached to the PCL/collagen scaffold and remained viable. Figure 3 shows representative images of ECs attached to each scaffold group. The cells formed a confluent monolayer on the surface of the 100-nm and 300-nm scaffolds; however, cells on the 1200-nm scaffolds appeared to have infiltrated into the constructs (white arrows, Figure 3) evidenced by fibers traversing over the fluorescent cells, which were located at an

imaging focal plane within the scaffold. Furthermore, cells on 100-nm and 300-nm aligned scaffolds displayed an elongated morphology, whereas cells on random scaffolds exhibited a polygonal, cobblestone-like appearance. Quantitative analysis of the images indicated that projected cell area on the 1200-nm scaffolds was significantly diminished compared to the 100-nm and 300-nm scaffolds, whereas cell area was not significantly affected by degree of fiber alignment (Figure 4a). Although fiber alignment did not have a statistically significant impact on projected cell area, it did have a dramatic effect on cell elongation, as measured by cell aspect ratio and length of long axis. Figures 4b,c show that cells on the 100-nm and 300-nm aligned scaffolds had significantly greater aspect ratios and long axis lengths as compared to those on 1200-nm and random scaffolds.

Figure 3 shows that fiber orientation had a strong impact on guiding cell alignment on 100-nm and 300-nm scaffolds, whereas cells on 1200-nm scaffolds were unaffected. Normalized histograms of cell orientation on 100-nm and 300-nm scaffolds provided quantitative evidence that cell alignment increased with greater fiber alignment (Supplemental Figure 2). Furthermore, cell angular deviation systematically decreased with diminishing fiber angular standard deviation for these scaffold groups (Table 1). In contrast, the analysis demonstrated that fiber alignment did not impact cell orientation on the 1200-nm scaffolds.

3.3. Structural and cell-substrate protein expression and organization

Immunofluorescence staining of cells on electrospun scaffolds was performed to determine the effect of scaffold topography on expression and organization of two principal structural proteins (F-actin and VE-cadherin) and a cell-substrate linker protein (vinculin). Staining for F-actin showed that cells on 100-nm and 300-nm random scaffolds had randomly oriented actin filaments that appeared to line the periphery of the cells (Figure 5). In contrast, cells on 100-nm and 300-nm fully-aligned scaffolds exhibited thick actin filaments traversing the full length of the cell parallel to fiber alignment. Cells on the 1200-nm scaffolds also stained positive for F-actin, however, the actin appeared to be weakly expressed. All 100-nm and 300-nm scaffolds showed strong staining for VE-cadherin at cell-to-cell contact regions, whereas this protein was not expressed in cells on 1200-nm scaffolds (Figure 6). To determine the effect of scaffold topography on cell-scaffold interactions, ECs were stained for vinculin, an important protein complex for cell adhesion. Figure 7 shows that cells on semi-aligned and fully-aligned scaffolds displayed higher levels of vinculin as compared to those on random scaffolds. The total area occupied by vinculin in the images was quantified using image analysis and results are shown in Figure 8 ($n = 3$ scaffolds per condition). Cells on semi-aligned and fully-aligned scaffolds exhibited 2–3 times the amount of vinculin expression of those cells on random scaffolds; however, no statistically significant difference in amount of vinculin was observed between cells on semi-aligned versus fully-aligned scaffolds.

3.4. EC adhesion under fluid flow

Confluent monolayers of ECs on both 100-nm and 300-nm scaffolds were exposed to continuous hydrodynamic shear stresses of 20 and 40 dyne/cm² using a PPFC to test the adhesion strength of ECs on different scaffold topographies. Cells on 1200-nm scaffolds were not exposed to flow since confluent monolayers had not formed and therefore were not considered as viable for use as a vascular graft lumen. Cell adhesion was dynamically measured by counting cell nuclei on the scaffolds for a duration of 60 min under flow. Figure 9a shows the percentage of cells attached to the 100-nm and 300-nm scaffolds at a shear stress of 20 dyne/cm² as a function of time. The rate of cellular detachment was the greatest for cells on the random scaffolds as compared to those on the aligned scaffolds. When cells were exposed to 40 dyne/cm² (Figure 9b), the rate of cellular detachment increased for cells on all scaffolds as compared to those exposed to 20 dyne/cm² (Figure 9a),

however the trends remained the same between scaffold groups. Figures 9a and 9b show that cell detachment from all scaffolds at both shear stresses normalize after about 40 min. Figure 9c shows the percentage of cells attached on all scaffolds after 60 min flow at both shear stresses. These results demonstrate that ECs exposed to 20 dyne/cm² on fully-aligned scaffolds display roughly 95% cell adherence, whereas only ~80% and ~60% of cells remained adherent on semi-aligned and random scaffolds, respectively. At a shear stress of 40 dyne/cm², roughly 62% of cells remained adherent on fully-aligned scaffolds, whereas 46% and 27% of cells were adherent to semi-aligned and random scaffolds, respectively. The results also demonstrate that scaffold fiber diameter did not have a statistically significant impact on cell attachment after 60 min of fluid flow.

4. Discussion

Due to the prevalence of vascular disorders, such as coronary and peripheral artery disease, there exists a great need for viable conduits for bypass grafting that overcome the drawbacks associated with the use of autologous grafts. Vascular tissue engineering has provided a promising approach for creation of such grafts, commonly in the form of tubular scaffolds composed of natural or synthetic materials wherein viable vascular cells are seeded to allow construct remodeling once implanted *in vivo* [Ju et al., 2010, L'Heureux et al., 2006, L'Heureux et al., 2007, Lee et al., 2008, Lee et al., 2007]. A critical factor for the success of these bioengineered vessels is the formation of a well-functioning endothelium on the lumen portion of the construct that provides the graft with a non-thrombogenic layer between circulating blood and the arterial wall. The endothelium should be well adhered to the surface of the construct and exhibit native EC morphology, specifically, ECs aligned with the direction of blood flow to permit high shear stress resistance and minimal flow disturbance [Aird, 2007, Aird, 2007, Inoguchi et al., 2007, Mott et al., 2007]. We hypothesized, therefore, that if ECs can be directed to adopt morphology, alignment, and F-actin organization similar to those *in vivo* using scaffold topographical cues, then resistance to deformation and detachment under shear stress may be enhanced as a result. Therefore, the main objective of this study was to examine the effect of scaffold design parameters, such as fiber diameter and orientation, on EC morphology, alignment, and adherence to the surface under fluid flow. The overarching goal was to investigate the potential of these electrospun substrates to direct ECs to form a confluent endothelium with morphological and structural protein properties similar to ECs *in vivo* in an effort to increase adherence to the scaffold surface.

To test this hypothesis, PCL/type I collagen scaffold topography was varied by tuning electrospinning parameters and mandrel rotation rates to systematically produce a 3 x 3 scaffold property matrix consisting of 3 different fiber diameters (roughly 100 nm, 300 nm, and 1200 nm) each with varying degrees of fiber alignment. PCL/collagen was specifically chosen for this study because of its promising characteristics as a vascular graft that include excellent mechanical properties [Lee et al., 2008], capacity to facilitate EC attachment/growth [Ju et al., 2010], and patency when implanted *in vivo* [Tillman et al., 2009]. Quantitative analysis of SEM images show that the degree of fiber alignment systematically increased with greater mandrel rotation rate (Figure 3), while the angular standard deviation systematically decreased (Table 1), indicating a more aligned structure. Scaffold topography had a large impact on EC morphology as visualized by fluorescence microscopy of Calcein AM stained cells. Cells had a well-spread morphology and were able to form confluent monolayers on 100-nm and 300-nm scaffolds, whereas cells on 1200-nm scaffolds were rounded and appeared to have infiltrated into the scaffold (Figure 4). In addition, ECs on 100-nm and 300-nm scaffolds expressed VE-cadherin in a continuous and linear manner at the periphery of cells where cell-cell contacts occurred, regardless of fiber orientation, indicating a confluent endothelium (Figure 8). Quantitative analysis of the fluorescent

images indicated that cell area and length of long axis were significantly greater for cells on 100-nm and 300-nm scaffolds as compared to 1200-nm scaffolds (Figures 5b and c). These findings are consistent with a study by Ju *et al.*, in which ECs displayed a well-spread morphology only on 270 nm diameter PCL/collagen fibers when compared to cells on micron diameter fibers [Ju *et al.*, 2010]. One possible explanation is that an increase in pore size, which is known to substantially increase with larger electrospun fiber diameter [Ju *et al.*, 2010, Pham *et al.*, 2006], may lead to a lack of sites available for focal adhesions to form on the underlying substrate and thus prevent adequate cell spreading. Nonetheless, electrospun meshes fabricated in this study (i.e. 100-nm and 300-nm) allowed EC spreading and formation of an uninterrupted endothelial layer, as evidenced by VE-cadherin expression at cell-cell junctions, after 3 days post-seeding.

Our results show that fiber orientation had a significant impact on directing the degree of EC alignment and elongation – consistent with the phenomenon of contact guidance. Similar studies have shown that various cell types, such as fibroblasts [Bashur *et al.*, 2006], neural stem cells [Lim *et al.*, 2010], tendon progenitor cells [Yin *et al.*, 2010], and smooth muscle cells [Choi *et al.*, 2008], respond to aligned fiber matrices in a similar manner by elongating and aligning parallel to fiber orientation. For this study, a systematic increase in EC alignment coincided with a greater degree of fiber alignment (Figure 6), while the angular standard deviation for both cells and fibers systematically decreased (Table 1). Additionally, ECs were elongated in the direction of fiber orientation for 100-nm and 300-nm aligned scaffolds quantified by a significant increase in cell aspect ratio and long axis (Figures 5b and c). Concurrent with variations in cell alignment and elongation, ECs displayed drastically different cytoskeletal arrangements in response to scaffold topography. ECs on 100-nm and 300-nm random scaffolds exhibit a banding pattern of F-actin at the periphery of the cells, whereas cells on fully-aligned 100-nm and 300-nm scaffolds display F-actin bundles that traverse the entire length of the cell parallel to fiber orientation (Figure 7). These results are strikingly similar to those in a study by Inoguchi *et al* [Inoguchi *et al.*, 2007], where HUVECs were seeded on the lumen portion of an electrospun vascular graft and exposed to gradually graded shear stress over a 3 day period. In that study, HUVECs exposed to incremental shear stress aligned with the direction of flow and expressed thick bundles of F-actin parallel to shear stress, whereas cells under static conditions retained a cuboidal morphology and expressed banding patterns of F-actin at their periphery. When exposed to fluid flow, ECs displaying a cuboidal morphology detached in response to shear stress less than 3.2 dyne/cm², whereas cells that had gradually undergone a regimen of gradual increase in shear stress over a 3 day period displayed an aligned morphology and remained adherent under a shear stress of 19.2 dyne/cm² [Inoguchi *et al.*, 2007]. This is one example of how EC adherence to a scaffold can be improved *in vitro* by using dynamic culturing conditions such as exposing EC-seeded vascular grafts to physiological fluid flow through bioreactor preconditioning [Baguneid *et al.*, 2004, Williams *et al.*, 2004]. Previous work has shown that preconditioning grafts before implantation increases EC retention, functionality, and graft patency once implanted *in vivo* as compared to non-conditioned grafts [Dardik *et al.*, 1999, Yazdani *et al.*, 2010]. As a result of shear stress preconditioning, ECs adopt an elongated morphology, align with the direction of fluid flow, and display cytoskeletal (F-actin) and adherens junction protein (vascular endothelial (VE) cadherin) organization similar to the native endothelium [Inoguchi *et al.*, 2007, Yazdani *et al.*, 2010]. These morphological and structural protein properties directly contribute to an endothelium with minimal flow disturbance and high adherence to the graft [Aird, 2007, Aird, 2007, Inoguchi *et al.*, 2007, Mott *et al.*, 2007] – both of which are required for a successful vascular graft. While shear-stress preconditioning is an effective method to increase the attachment and shear resistance of seeded ECs, this process is resource intensive and time consuming, often taking several days or weeks for the ECs to reorganize sufficiently to resist

detachment under physiological shear stresses (20 – 40 dynes/cm²) [Niklason et al., 1999, Quint et al., 2011].

In contrast, the method used in this study to influence endothelial cell morphology did not require the use of fluid flow preconditioning to increase EC adherence to the scaffolds under physiological shear stress. We found that topographical guidance of ECs into an elongated morphology and organized cytoskeleton in the direction of fluid flow was an effective means to increase EC resistance to detachment under fluid flow. Specifically, we demonstrated that ECs cultured on fully-aligned scaffolds had roughly 35% more cells adherent to the scaffold as compared to ECs on random scaffolds after 60 min of flow at both shear stresses of 20 and 40 dyne/cm². In addition, ECs cultured on aligned scaffolds had a much lower rate of detachment compared to those cultured on random scaffolds (Figure 9b, c). With the increase in cell elongation, our results demonstrated that ECs also expressed higher levels of vinculin – an adaptor protein that is recruited from the cytoplasm to regulate focal adhesion complexes in anchorage dependent cells [Humphries et al., 2007]. Quantification of vinculin expression showed that ECs on semi-aligned and fully-aligned scaffolds had roughly 2–3 times the amount of vinculin as compared to ECs on random scaffolds. An interesting finding of this work is that even though ECs cultured on semi-aligned and fully-aligned scaffolds displayed statistically insignificant differences in morphologies (Figure 4) and vinculin expression (Figure 8), ECs on fully-aligned scaffolds had roughly 15% more cells attached after 60 min of flow at both shear stresses when compared to ECs on semi-aligned scaffolds. We propose that the difference in EC attachment between the semi-aligned and fully-aligned scaffolds arose from differences in cell alignment and F-actin organization with the direction of flow. In a study conducted by Barbee *et al* [Barbee et al., 1994], ECs cultured under flow conditions showed that cells had a significant decrease in height profile and were streamlined with the direction of flow as compared to those cultured under static conditions. The authors showed that cell alignment and elongation in the direction of flow decreased the shear stress and gradients of shear stress at the cell surface. We believe that a similar phenomenon occurred with cells on the fully-aligned scaffolds in the present study, where topography-induced elongation and alignment in the direction of fluid flow diminished shear stress at the cell surface, thereby decreasing detachment as compared ECs on random and semi-aligned scaffolds. Therefore, our findings indicate that not only can EC elongation, alignment, and F-actin organization be directed by tuning fiber orientation through contact guidance, but also the resulting changes in F-actin organization and increased adherence to the scaffold surface are similar to those of ECs exposed to hydrodynamic shear stress without the use of dynamic culturing conditions using fluid flow systems.

5. Conclusion

In this study, we varied the fiber diameter and orientation of electrospun fibers in an effort to elucidate the impact of scaffold topography on EC morphology, alignment, structural protein expression/organization and adherence under fluid flow. We found that ECs on electrospun scaffolds formed confluent monolayers and alignment of cells was found to systematically increase as a function of increased fiber orientation, leading to a fully aligned endothelium on the most aligned scaffolds. Concurrent with cell alignment, ECs exhibited thick bundles of oriented F-actin parallel to the direction of fiber alignment, mimicking F-actin organization of native ECs in straight artery segments that are exposed to high shear stress. ECs on fully-aligned scaffolds displayed higher levels of adherence to the scaffolds under physiological shear stress as compared to those on random and semi-aligned scaffolds. These findings indicate that EC morphology and alignment can be directed through tuning electrospun topography and could be used to form an uninterrupted, fully aligned and shear resistant endothelium for vascular tissue engineering applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative SEM images of electrospun PCL/collagen scaffolds. A 3 x 3 scaffold property matrix was formed by systematically varying fiber diameter (100-nm, 300-nm, and 1200-nm) and degree of fiber alignment (random, semi-aligned, and fully-aligned).

Figure 2. Fiber diameters for scaffolds electrospun from various polymer solution concentrations (5%, 10%, and 15% w/v) and fabrication parameters with varying degrees of fiber alignment. An “*” indicates a statistical difference from the 5% (w/v) group, and a “#” indicates statistical difference from the 10% (w/v) group ($p < 0.05$) ($n = 250$ fibers for each group).

Figure 3.

Representative images of Calcein AM stained ECs on electrospun scaffolds. Cells displayed a more cuboidal morphology on random electrospun scaffolds as compared to scaffolds with defined fiber alignment, where cells were elongated and aligned with the direction of fiber orientation. Cells on 1200-nm scaffolds displayed a rounded morphology, no preferred orientation, and appear to have infiltrated into the scaffold (white arrows).

Figure 4.

Quantitative analysis of a) cell area, b) aspect ratio, and c) length of long axis of Calcein AM stained ECs on electrospun scaffolds. Cell area was significantly greater on 100-nm and 300-nm scaffolds when compared to 1200-nm scaffolds. Cell aspect ratio and length of long axis were significantly increased on aligned 100-nm and 300-nm scaffolds as compared to random and 1200-nm scaffolds. An “*” indicates a statistical difference from the 1200-nm scaffold group, and a “#” indicates statistical difference from the random scaffold group ($p < 0.05$) ($n = 10$ images per group).

Figure 5.

Representative fluorescent images of cytoskeletal F-actin organization for ECs on scaffolds with varying fiber diameters and orientations. F-actin (red) was organized at the periphery of cells on random 100-nm and 300-nm scaffolds, but was located throughout the cells in thick bundles parallel to electrospun orientation on 100-nm and 300-nm fully-aligned scaffolds.

Figure 6.

Representative fluorescent images of VE-cadherin (green), showing that the cell-cell adhesion protein was strongly expressed at the periphery of cells where cell-cell contact occurred on 100-nm and 300-nm scaffolds. VE-cadherin was not, however, expressed in cells on 1200-nm scaffolds.

Figure 7. Staining for vinculin revealed that cells on aligned scaffolds expressed a considerably higher level of vinculin than cells on random scaffolds.

Figure 8.

The total area covered by vinculin in each image was quantified using image analysis. The results show that cells on aligned scaffolds expressed 2–3 times the amount of vinculin than cells on random scaffolds. A “#” indicates a statistical difference from the random scaffold group ($p < 0.05$) ($n = 9$ images per group).

Figure 9.

Percentage of cells that remained adherent to the 100-nm and 300-nm scaffolds during 60 min of a) 20 dyne/cm² and b) 40 dyne/cm² continuous hydrodynamic shear stress. c) Percentage of cells adherent to the scaffolds after 60 min of continuous hydrodynamic shear stress. Values marked with the same letter are not significantly different ($p < 0.05$)

Table 1

Electrospun fiber diameter, fiber angular standard deviation, and cell angular standard deviation for scaffolds fabricated with varying solution concentrations, electrospinning parameters, and mandrel rotation rates.

	Solution Concentration (% w/v)	Rotation Speed (rpm)	Designation	Fiber Diameter (nm)	Fiber Angular Deviation (°)	Cell Angular Deviation (°)
5	0		100-nm, Random	99 ± 39	68.7	59.8
	1500		100-nm, Semi-Aligned	127 ± 39	35.9	47.2
	3000		100-nm, Fully-Aligned	135 ± 44	27.9	33.5
10	0		300-nm, Random	326 ± 103	54.6	51.8
	1500		300-nm, Semi-Aligned	297 ± 100	34.7	43.5
	3000		300-nm, Fully-Aligned	311 ± 73	19.1	24.8
15	0		1200-nm, Random	1235 ± 205	56.6	55.5
	1500		1200-nm, Semi-Aligned	1101 ± 196	43.4	58.3
	3000		1200-nm, Fully-Aligned	1341 ± 296	20.4	59.4