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Aligned Nanofibrillar Collagen Regulates Endothelial Organization and Migration

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Abstract

Aims—Modulating endothelial cell (EC) morphology and motility, with the aim to influence their biology, might be beneficial for treatment of vascular disease. We examined the effect of nanoscale matrix anisotropy on EC organization and migration for vascular tissue engineering applications.

Materials and Methods—We developed a flow processing technique to generate anisotropic nanofibrillar collagen. Human ECs were cultured on aligned or on randomly oriented collagen, and their cellular alignment and cytoskeletal organization were characterized by immunofluorescence staining and time-lapse microscopy.

Results—ECs were elongated along the direction of aligned collagen nanofibrils and had organized focal adhesions. Cellular protrusion migrated with greater directionality and higher velocity along the anisotropic nanofibrils, compared to cells on random nanofibrils. The flow technique can be adapted to fabricate vascular grafts that support endothelial phenotype.

Conclusions—Aligned nanofibrillar collagen regulates EC organization and migration, which can significantly contribute to the development of vascular grafts.

Keywords

nanofibrillar collagen; endothelial cell; patterning; vascular graft; cell-matrix interactions; endothelial migration; anisotropy; cell patterning

1 Introduction

Cardiovascular disease is the major cause of morbidity and mortality in the United States [1]. Most cardiovascular disease is attributable to the effects of atherosclerosis, with myocardial infarction and stroke due to atherosclerotic plaque rupture, thrombosis and/or embolism. Notably, the distribution of atherosclerotic lesions in the circulation is not uniform. Lesions tend to form at sites of disturbed flow, e.g. bends, branches, and bifurcations [2]. At these sites, there is an early disturbance of normal endothelial functions, which represents the earliest pathological process in the development of atherosclerotic arterial disease [3].

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The endothelium is a delicate monolayer of cells lining blood vessels. A healthy endothelium controls vessel diameter by its production of vasodilator substances. Some of these substances, such as nitric oxide and prostacyclin, also inhibit the adhesion of platelets and leukocytes. These and other paracrine substances released by the endothelium prevent vascular thrombosis and inflammation [3]. By contrast, at sites of disturbed flow, the endothelium produces less of the homeostatic factors, and instead elaborates adhesion molecules and chemokines that promote the interaction of circulating blood elements with the vessel wall.

The morphology of endothelial cells (ECs) is a well-known indicator of EC phenotype. Elongated ECs with cytoskeletal elements aligned in the direction of blood flow correspond to a healthy, athero-resistant phenotype. This endothelial morphology is typically observed in straight segments of the arterial tree, where atherosclerotic lesions are less likely to develop. In contrast, ECs with cobblestone morphology and randomly oriented EC cytoskeletons are typically found at sites of disturbed flow, and are athero-susceptible [4]. After vascular injury or disease, EC migration is important in the angiogenesis process to form neovessels in the surrounding tissue. EC migration involves protrusion of filopodia and lamellipodia at the leading edge, forward movement of the cell body, and release of the lagging edge of the cell [5]. Therefore the ability to control EC morphology and motility, with the aim to influence EC biology, might be highly beneficial in the prevention or treatment of vascular disease.

Surfaces of patterned topography, with features in the micro or nanoscale range, have been widely used to investigate the behavior of cells. Nanopatterning, in the form of islands, lanes, or grooves, has successfully demonstrated the ability to influence both the morphology and migration of ECs [4–11]. Commonly used fabrication techniques include soft lithography, photochemistry, inkjet printing, or electrospinning. These techniques, however, may have limitations in achieving high resolution features, repeatability, translation to 3D surfaces, or expensive fabrication requirements [4]. We present a simple flow processing technique that extrudes aligned collagen fibrils with nanoscale topography. Unlike previous patterning methods used to regulate the cytoskeletal organization of ECs, the aligned collagen fibrils do not use physical or biochemical confinement to restrict the cell's motion, thereby better mimicking native extracellular matrices (ECMs). The aligned fibrils also provide a useful platform to investigate EC migration, where previous migration investigations have been limited to conditions using hemodynamic shear, chemotactic gradients, or physical channels [12,13].

In this study, we utilize a facile flow technique to fabricate aligned nanofibrillar collagen and then examine the effect of matrix nanopatterning on EC organization and migration. We demonstrate that parallel-aligned nanofibrillar collagen not only guides EC cytoskeletal reorganization, but also promotes increased cellular migration along the direction of the nanofibrils, which has therapeutic applications in the generation of aligned vascular grafts.

2 Materials and Methods

2.1 Fabrication of aligned collagen strips and grafts

High concentration rat tail collagen-Type I (BD Biosciences) was initially prepared at a stock concentration of 9–10 mg/mL in 0.02N Acetic Acid, pH 3.5 and dialyzed to 30 mg/mL using a previously published method [14]. Using a 1 mL plastic syringe with a 22 gauge blunt needle, the dialyzed collagen was manually deposited as thin strips of collagen onto a glass slide (Fig. 1A). The syringe moved at an approximate rate of 340 mm/s, while the collagen was extruded at 3.2 mL/min. In contrast, strips of non-aligned collagen fibrils, or the randomly oriented control, were produced by moving the syringe at a significantly

slower velocity, 50 mm/s. The slow syringe velocity was also coupled with a slow extrusion rate, 0.02 mL/min, to produce strips of random collagen with similar thickness and width as the aligned collagen strips. The glass slide and the needle were submerged in 37°C, 10X-phosphate buffered saline (PBS), pH 7.4, during the procedure in order to initiate fibrillogenesis immediately as the collagen was deposited. The collagen completely dried under a laminar flow hood overnight and rinsed several times with MilliQ water afterwards. The collagen strips remained adhered onto glass slides (Fig. 1A). This method produces randomly oriented collagen strips that are $\sim 1.1 \times 0.8 \times 30$ mm and aligned collagen strips that are $\sim 1.0 \times 1.2 \times 30$ mm.

In order to produce a three-dimensional aligned collagen conduit for potential use as a vascular graft, the collagen was extruded using an aluminum syringe in a plunger motion, as described previously [14]. The collagen grafts then received a cross-linking treatment of 50 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride, EDC, (Pierce) in 100% ethanol, agitated overnight at room temperature. The EDC solution was then aspirated and the grafts were placed in a vacuum desiccator for 2 hours or until the collagen was completely dehydrated. In preparation for cell culture or mechanical testing, the grafts were rehydrated in PBS.

2.2 Scanning Electron Microscopy (SEM)

Samples were processed for routine SEM, according to previous publications [15], using a Hitachi S-3400N VP scanning electron microscope (Hitachi Ltd).

2.3 In vitro culture experiments

Primary human dermal microvascular ECs were grown in EGM2-MV (Lonza) media. Prior to *in vitro* studies, the collagen substrates were sterilized in 70% ethanol and then incubated in PBS before cell seeding. ECs were plated at a seeding density of 23–28k cells/cm² and incubated for 24 hours (collagen strips) or 72 hours (vascular grafts).

2.4 Immunofluorescence staining

At indicated time points, samples were fixed in 4% paraformaldehyde. Samples were immunofluorescently stained using antibodies against CD31 (Dako Cytomation), Ki67 (DakoCytomation), or paxillin (BD Transduction Labs), according to previous publications [16,17]. In addition, phalloidin (Invitrogen) was used to visualize the F-actin assembly. All nuclei were visualized by Hoechst33342 dye and imaged on a Nikon fluorescence microscope or a Leica SP2 Acousto-Optical Beam Splitters confocal microscope.

2.5 Analysis of cellular alignment and proliferation

Cell alignment was measured, using ImageJ (NIH, v.1.42), as the angle formed by the cell's principal axis, relative to the direction of the collagen nanofibrils, where a minimum angle of 0° denoted parallel alignment from the axis of the nanofibrils, and a maximum of 90° suggested perpendicular alignment [16]. For quantification of randomly oriented fibrils, an arbitrary axis was selected. Proliferation was defined as the percentage of total nuclei expressing the proliferation marker Ki67. The cell shape index (CSI) was equivalent to $(4\pi \cdot \text{Area})/(\text{Perimeter}^2)$, where the value of 1 approximated the shape of a circle and a value of 0 depicted that of a straight line [18]. Cells cultured on aligned or random collagen were imaged in five representative fields, and 25 cells in each field were evaluated. The data are shown as mean $\pm$ standard deviation ($n = 3$). A Student's *t*-test was compared between two groups, and significance was accepted at $P < 0.05$. We also used two-dimensional (2D) fast Fourier transform (FFT) analysis (Image J) to further characterize alignment of the collagen nanofibrils and cellular alignment [19]. The orientational order of the cells is quantified by

an order parameter, denoted by S , where $S = \langle (3\cos^2\theta - 1)/2 \rangle$ [20]. A perfectly aligned distribution where the cells have an alignment angle, θ , of 0° relative to the collagen axis would indicate $S=1$, and $S=0$ suggests a random distribution of orientation angles.

2.6 Time-lapse microscopy and cell tracking analysis

A Zeiss inverted microscope with a motorized stage was used to conduct a time-lapse study of the ECs. The microscope was enclosed in a plexiglass chamber and maintained a 37°C , 5% CO_2 environment. After 30 minutes of incubation on the collagen matrices, a $10\times$ objective was used to obtain phase contrast images every 2.5 minutes until 24 hours elapsed since the seeding of the cells. The movement of the cells was manually tracked by recording the coordinates of the tip of the cell's protrusion on its leading edge using ImageJ's plug-in, MTrackJ (v.1.4.1). $N=10$ cells on either the aligned or random collagen were observed. The images were oriented so that the x-axis was parallel to the collagen fibril direction. An arbitrary axis was chosen for the randomly oriented collagen. Tracking began when a cell protrusion on the leading edge of a cell appeared and completed when the protrusion was no longer present or when the time-lapse ended. A protrusion of the cell was identified as any thicker structure of the cell that extends, which can include lamellipodia, pseudopodia, filopodia and microvilli, similarly defined in the time-lapse analysis of Fujita et al [21]. Using the Chemotaxis and Migration Tool plug-in (v.1.01, ibidi), the recorded coordinates were used to produce vector plots and Rose diagrams depicting cell motility. The coordinates of the cells were also used to analyze the mean squared displacement (MSD), where $MSD(t) = \langle [x(t+t_0) - x(t_0)]^2 + [y(t+t_0) - y(t_0)]^2 \rangle$. To better emphasize the anisotropic movement of the aligned cells, the displacement was decoupled into an x and y direction where $MSD(t) = MSD_x(t) + MSD_y(t)$, or $MSD_x(t) = [x(t+t_0) - x(t_0)]^2$ and $MSD_y(t) = [y(t+t_0) - y(t_0)]^2$. The MSD analysis focused on the time range from 6–12 hours of elapsed time after seeding to allow sufficient time for the cells on aligned collagen to elongate. Velocity was calculated as the total accumulated distance divided by the elapsed time. Velocity could also be decoupled into the x and y direction, where the velocity in the x direction was $v_x = (\sum |x_i - x_{i-1}|) / (t_n - t_0)$ for $i = 1$ to n . V_y was defined similarly.

2.7 Mechanical characterization of collagen vascular grafts

Elastic modulus and burst pressure were two measurements used to characterize the mechanical integrity of the collagen vascular grafts. An Instron 5844 mechanical tester, using a $\pm 10\text{N}$ load cell, was used to measure the elastic modulus of the collagen vascular grafts at a constant strain rate of 15 mm/min . To measure burst pressure, a pressure transducer (Cole-Parmer) connected to LabView (v.8.5) was able to measure the increasing pressure response of the vascular graft while water was injected into the conduit at a rate of 1.0 mL/min . Burst pressure was defined as the maximum pressure reached by the system until the graft experienced a mechanical failure. Details of both procedures have been discussed previously. $n = 5$ were measured for both mechanical measurements.

3 RESULTS

3.1 Production of aligned nanofibrillar collagen matrices

The extrusion flow processing technique was successfully able to produce strips of uniformly aligned collagen fibrils. With a syringe velocity of 340 mm/s and extrusion flow rate of 3.2 mL/min , the hydrodynamics of the flow was able to force the collagen fibrils to align along the direction of flow. A schematic of the technique is depicted in Fig. 1A, where the syringe velocity was defined as v_s and v_c was the collagen velocity. The syringe moved in the opposite direction of the extruded collagen, allowing the collagen solution to anchor at one end and be drawn in a line along the glass slide. Depositing the collagen solution into 37°C , $10\times\text{-PBS}$ allowed flow-directed orientation and fibrillogenesis to occur

simultaneously in the collagen solution. As demonstrated in the SEM images (Fig. 1B), the flow procedure was capable of producing matrices of highly aligned collagen fibrils. In contrast, extruding the collagen at a significantly slower velocity produces a matrix composed of randomly oriented collagen fibrils. The flow technique produced collagen fibrils in the nanometer scale range, where the average fibril diameter was approximately 45 nm in both the aligned and random collagen matrices. Alignment of the fibrils was quantified by 2D FFT analysis (Fig. 1B–C). The inset of Fig. 1B depicts the frequency spectrum of the collagen fibrils, in which random orientations are presented as white pixels uniformly distributed about the center, whereas parallel-aligned fibrils are represented as white pixels organized along the direction of the nanofibrils. The frequency spectrum can be further quantified as a 2D FFT alignment plot, in which parallel-aligned nanofibrils are depicted to have two distinct peaks spaced 180° apart, whereas randomly oriented nanofibrils show only low-frequency peaks (Fig. 1C). Therefore 2D FFT analysis confirms high alignment of the aligned collagen nanofibrils.

3.2 Anisotropic collagen fibrils modulate EC organization

We cultured ECs on the random or aligned nanofibrillar collagen strips and assessed the matrix-induced cellular morphology. The ECs elongated and organized their actin cytoskeleton along the direction of the aligned collagen fibrils, as depicted by phalloidin staining. By contrast, in those cells grown on the randomly oriented collagen, actin assembly was randomly distributed (Fig. 2A–B). The cellular alignment was quantified by the absolute degree of alignment, in which cells that were aligned along the direction of the collagen fibrils had a value close to 0° and cells perpendicular to the collagen axis would have a value of 90°. As shown in Fig. 2C, the mean degree of alignment of the EC on aligned collagen was $27.1 \pm 9.9^\circ$, which was significantly different from the mean degree of alignment of $41.4 \pm 3.4^\circ$ on the randomly oriented collagen fibrils ($P < 0.05$). As shown by the frequency distribution plot in Fig. 2D, the majority of cellular alignment was within 30° from the axis of the aligned collagen fibrils. Cellular alignment was further verified using 2D FFT, in which it demonstrated that the cells on aligned collagen had two distinct peaks that were 180° apart, suggesting high cellular alignment (Fig. 2E). In contrast, the cells cultured on randomly oriented nanofibrillar collagen did not show any distinctive peaks, suggesting the lack of spatial organization. The 2D FFT trend correlates with the differences in order parameter, S , where ECs on aligned collagen demonstrated a higher orientational order with a value of S closer to 1 than cells on random nanofibrils (0.62 ± 0.20 vs. 0.32 ± 0.06 ; $P < 0.05$). Cytoskeletal organization was also assessed by the CSI values, as cells on the aligned collagen substrates had a significantly lower mean CSI value (0.599 ± 0.014 vs. 0.664 ± 0.020 ; $P < 0.05$).

Besides developing organized cytoskeletons, the ECs on the aligned substrate were also characterized by organized focal adhesions. As shown by immunofluorescence staining, the focal adhesion protein paxillin was predominantly localized along the front and back ends of the cells, whereas on the randomly oriented substrates the paxillin protein was distributed uniformly throughout the cell (Fig. 2F). Proliferation based on the expression of the cell cycle antigen, Ki67 ($40 \pm 10\%$ random vs. $36 \pm 2\%$ aligned) was not significantly different. These results demonstrate that the anisotropic collagen nanofibrils could organize the actin cytoskeleton, cell shape, and focal adhesions.

3.3 Anisotropic collagen nanofibrils direct EC migration

A time-lapse microscopy study was conducted to evaluate how the ECs would respond to an anisotropic, underlying surface as they were cultured on the collagen strips for 24 hours. The development of matrix-induced alignment of the ECs is depicted in the images in Fig. 3A. ECs seeded onto a randomly oriented collagen matrix demonstrated no distinct orientation

and remained circular-shaped throughout the course of the time-lapse. However, the cells seeded onto aligned collagen fibrils gradually responded to the anisotropy of the underlying surface by demonstrating a distinct elongated and polarized morphology along the fibril direction within 4 hours of seeding. The dynamics of the cell response could be further evaluated by manually tracking the location of a cell protrusion on its leading edge as time elapsed. In Fig. 3B, vector plots and rose diagrams (inset) were used to display the coordinates of the cell protrusions over time. The protrusions of cells on a random matrix moved at all angles, with no directionality, as indicated by its random trajectory on the vector plot. The movements of a cell's protrusions on an aligned matrix, however, were dominated along the direction of the underlying collagen fibrils. The rose diagrams further highlighted this behavior by demonstrating the higher frequency of coordinates along the x-axis for a cell on aligned collagen, whereas the rose diagram of the random matrix was more isotropic.

Although MSD is often used to relate cell migration to a random walk model, we utilized this method to assess the anisotropy in cell movement. To emphasize the difference in directionality, the MSD was decoupled into movements along either the x or y axis. By plotting the sum of the square of the x ((MSD_x)) or y ((MSD_y)) coordinates versus time, the response indicated how a cell moved in the direction of these respective axes over time. The graphs in Figs. 3C reflect the MSD_x and MSD_y for a representative cell. Among all cells analyzed ($n=10$), the MSD_x was significantly higher on the aligned collagen ($1.1 \pm 1.7 \text{ mm}^2$) when compared to that on the randomly oriented sample ($0.30 \pm 0.54 \text{ mm}^2$, $P<0.01$). Similarly, cells on the aligned collagen showed a greater MSD_y ($0.09 \pm 0.30 \text{ mm}^2$) when compared to those grown on the random collagen ($0.05 \pm 0.14 \text{ mm}^2$, $P<0.01$). The large standard deviations in MSD are due to the varying lifetimes of the protrusions for the ten cells.

The velocity of the cell's protrusion on its leading edge was also regulated by the anisotropy of the collagen fibrils. Although the overall velocity was similar on both the aligned and randomly oriented collagen ($1.85 \pm 0.43 \text{ } \mu\text{m}/\text{min}$ vs. $1.81 \pm 0.34 \text{ } \mu\text{m}/\text{min}$, respectively), we found significant differences when the velocity vector was decoupled to the x (parallel to aligned nanofibrils) or y (orthogonal to aligned nanofibrils) direction. The v_x was 77% faster than v_y ($1.80 \pm 0.44 \text{ } \mu\text{m}/\text{min}$ vs. $0.42 \pm 0.07 \text{ } \mu\text{m}/\text{min}$, respectively; $P<0.001$) for aligned cells along the collagen fibrils, suggesting that the cells migrated more avidly along the direction of the collagen fibrils (Fig. 3D). In contrast, the cells on randomly oriented collagen migrated only 24% faster along the x direction ($1.43 \pm 0.29 \text{ } \mu\text{m}/\text{min}$, v_x vs. $1.09 \pm 0.27 \text{ } \mu\text{m}/\text{min}$, v_y ; $P<0.05$). The propensity of cells to migrate faster along the x direction on random oriented collagen may be due to the dimensions of the strips that favor overall migration longitudinally along the strip, although our results clearly demonstrate a faster velocity (v_x) of cell movement in the presence of matrix anisotropy.

3.4 Mechanical characterization of aligned collagen vascular graft

We applied our findings of nanotopography-induced EC alignment towards the generation of aligned collagen vascular grafts. By modifying the flow technique of extruding aligned collagen matrices in the presence of an annulus (Fig. 4A), we could extrude a three-dimensional, hollow conduit composed of aligned collagen fibrils (Fig. 4B). To improve the mechanical properties of the graft, we treated the grafts to a cross-linking treatment overnight with 50 mM EDC in 100% ethanol, followed by a dehydration step. The grafts are then rehydrated prior to mechanical studies. This final processing step of dehydration led to significant improvements in both the collagen graft's elastic modulus and burst pressure. As demonstrated in Fig. 4C–D, both methods of testing involved applying stress to the graft until mechanical failure was experienced. The post-treatment steps rendered the collagen grafts stronger, achieving an elastic modulus of $3.6 \pm 1.9 \text{ MPa}$ and a burst pressure of $900 \pm$

170 Torr, which is substantially stronger compared to our previous fabrication methods [14]. This data suggests that the flow technique was able to produce an aligned vascular graft with physiologically relevant mechanical properties.

3.5 Endothelial morphology on the aligned nanofibrillar vascular graft

To demonstrate the potential of the collagen conduit as a biological vascular graft, we examined the behavior of ECs on the anisotropic nanofibrillar graft after allowing the ECs to incubate for 72 hours on the scaffold. The ECs were highly elongated and aligned along the direction of the collagen fibrils, as visualized with SEM (Fig. 5A–B). The cells maintained their phenotype by expressing the endothelial cell adhesion molecule, CD31, which was intensely expressed at intercellular junctions (Fig. 5C). Furthermore, the cells continued to proliferate, as visualized by the nuclear-localization proliferation antigen, Ki67. These results suggest that the aligned vascular conduit could support endothelial attachment, phenotype, and proliferation.

4 DISCUSSION

The salient findings of this work are 1) extrusion flow processing is a facile and reproducible method for fabricating anisotropic nanofibrillar collagen scaffolds; 2) aligned nanofibrillar collagen organizes endothelial morphology in the assembly of focal adhesion proteins and cytoskeleton; 3) aligned nanofibrillar collagen modulates EC migration speed and direction; and 4) aligned collagen vascular grafts are mechanically stable and support EC alignment and phenotype.

Our flow processing method overcomes some of the limitations of other methods to induce geometric patterning of ECMs. Micropatterning, which includes techniques such as soft lithography, photochemistry, and inkjet printing, is a commonly used approach to influence cell morphology by employing physical confinement or biochemical cues. Soft lithography is inexpensive and can produce high resolution features but repeatability and translation to 3D surfaces are current limitations. High reproducibility can be achieved through other methods such as photochemistry or inkjet printing. However, photochemistry requires specialized and expensive instrumentation, while inkjet printing is relatively simpler but has been limited to features of low resolution (30–100 μm) [4]. Other alternative techniques to micropatterning produce anisotropic materials that may be more directly adaptable as tissue engineering scaffolds. Electrospinning has been used to induce the alignment of polymeric, nanometer-scale filaments in order to create scaffolds with similar structural properties of the native ECM [22,23]. The major disadvantages to this technique are the use of complex equipment, such as an electric field or high strength magnet, and relatively long fabrication times [24]. In addition, electrospun fibers can often have diameters in the hundreds of nanometer to micron scale, which is near the upper limit of the ECM fiber dimensions (50–500 nm) [24–26].

The molecular structure of collagen is a rod-like molecule consisting of three polypeptide strands wound in a left-handed helix. The collagen molecules self-assemble to form a hierarchy of fibril bundles, a process known as fibrillogenesis. In nature, these bundles of collagen fibrils make up the connective tissue of tendons and ligaments. The extrusion technique developed in the current work applied hydrodynamic flow to align the collagen in its monomeric state (present at low pH) during the flow process and then induced fibrillogenesis with an increase in pH. The collagen orientation was influenced by both a shear flow component, due to extrusion out of the syringe, and also an extensional flow component, as the syringe stretched the collagen along the glass slide. Because the syringe moved in the opposite direction of the extruded collagen, the extensional flow component was emphasized as the collagen solution adhered to the glass surface and the syringe was

drawn away from the anchoring point. The draw ratio, v_s/v_c where v_s is the syringe velocity and v_c is the linear collagen velocity, demonstrates the relative effect of modifying either the syringe or collagen velocity [27]. By changing the velocities, the flow technique produced either aligned or randomly oriented collagen due to different draw ratios, approximately 1 for aligned and 20 for the random control. The molecular order and orientation of the collagen was then stabilized by inducing fibril formation by extruding into a neutral pH PBS. Earlier techniques of preserving the supramolecular order of collagen structures involved exposure to ammonia vapor [28]. Electrostatic and hydrophobic interactions are usually the main factors in determining fibril diameter due to their role in fibril self-assembly, where pH, ionic strength, and collagen concentration have been known to affect fibril diameter [29,30]. As confirmed by the SEM images in Fig. 1B, the process of fibrillogenesis was able to preserve the flow-induced anisotropy of the collagen, suggesting that the fibrillogenesis occurred at a faster time scale than the relaxation of the collagen molecules. The flow-aligned collagen fibrils had dimensions on the nanoscale level, making the structure of these anisotropic nanofibrillar matrices directly comparable to the length scale of extracellular matrix components.

The broader impact of the flow technique described in this study is its ability to adapt and fabricate a three-dimensional conduit of collagen fibrils, a more biologically relevant shape for vascular applications. Although the main focus of this study was to evaluate EC interactions on anisotropic matrices of collagen nanofibrils, a logical extension of the cell-matrix interactions study is to demonstrate the functionality of the vascular graft. In addition to its nanoscale dimensions, the anisotropic conduit of nanofibrillar collagen manifested mechanical properties of physiological relevance. The collagen conduits were able to achieve a higher elastic modulus (3.6 ± 1.9 MPa) than what has been reported for a healthy human coronary artery (1.4 MPa) [31]. The graft's mechanical integrity along its radial dimensions was evaluated with its burst pressure (900 ± 169 Torr) which is significantly higher than the range of normal arterial pressures (80–120 Torr). The strong mechanical integrity of the vascular grafts is most likely attributable to the cross-linking and desiccation, rather than the orientation of the collagen fibrils. After desiccation and subsequent rehydration of the samples, the vascular conduits are visibly smaller in size, providing a visual confirmation that the tubes have significantly dehydrated. The post-treatment process of the grafts enhances the mechanical integrity of the collagen conduits by reducing the pore size, and thereby increasing the cross-link density, of the collagen structure. Cross-link density, N , and elastic modulus, E , are directly proportional, as defined by the rubber elasticity theory $N = E/RT$ where R is the gas constant and T is absolute temperature. SEM and confocal microscopy were then able to confirm that the aligned vascular conduit could support the attachment, phenotype, and proliferation of ECs. The strong structural integrity and positive cell-matrix interactions of the aligned collagen conduit demonstrated its significant potential for use in vascular applications.

The structural topography of these flow-induced anisotropic collagen fibrils allows a unique opportunity to evaluate how the morphology of ECs is regulated in response to an underlying matrix. In particular, the time-lapse analysis of EC protrusion motility on our anisotropic collagen matrices provides a different perspective than previous EC migration research. Time-lapse microscopy has primarily observed the migration of ECs on micropatterned lanes or nanogrooved substrates [4,6–9,21]. Although the studies have contributed greatly to the knowledge of cell migration, the previously used techniques employ the use of barriers to force cell alignment for anisotropic migration studies. These barriers, either in the form of physical (i.e. grooved channels) or biochemical (i.e. cytophilic and cytophobic areas to localize cell attachment), essentially constrict the cell to align by reducing their degrees of freedom and thereby confining their movements to limited areas. The aligned collagen nanofibrils used in this study, however, present an anisotropic matrix

to guide the organization of cytoskeleton assembly and induce cellular alignment without methods of confinement. The collagen matrix permits migration in any direction, but the anisotropic topography favors directionality of cell migration. This was observed by the preferential movement of the cellular protrusions along the collagen fibril axis and the faster probing velocity along the fibril, or x , direction ($v_x > v_y$). Li et al. observed a similar trend with migration velocity in bovine aortic ECs, where the cells migrated faster along the direction of micropatterned lanes coated with collagen [6]. Our study also used MSD, normally used to model cell movement as a random walk, as an alternative method to analyze the directionality of cell migration. The MSD analysis of the time-lapse studies revealed that the cytoskeletal alignment of ECs correlated with protrusion motility. The significance of this rapid and directional cell migration along the collagen nanofibrils may be especially important in the development of vascular grafts, where anisotropic migration might enhance the endothelialization process from the site of anastomosis [9].

There is increasing evidence that cells respond to substrate nanotopography through integrin transmembrane receptors, which relay external cues to the focal adhesion complex, which in turn connects to the intracellular actin cytoskeleton [32]. The cytoskeleton is a polymer network consisting of actin, microtubules, and intermediate filaments, and is required for cell protrusion, adhesion of the leading edge, and translocation of the cell body [33]. Once the cell adheres to its substrate, the cell begins to explore and probe its surroundings by movement of the filopodia and lamellipodia. Filopodia acts as tethering points for cell movement as the cell probes its environment, which is subsequently followed by the formation of focal adhesions and extension of cell protrusions [34].

Endothelial cells 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 manifest biological properties that promote atherosclerosis [3]. Intriguingly, in the human arterial circuit, there appear to be sites of focal senescence where endothelial cells are non-aligned. In the human iliac artery, endothelial cells at the bifurcation exhibit shorter telomeres [35]. Senescent human endothelial cells synthesize less nitric oxide, generate more superoxide anion, express adhesion molecules, and are less capable of replication and re-alignment in response to flow; these abnormalities can be reversed by restoring telomere length [36]. Accordingly, it may be important to consider endothelial alignment in maintaining endothelial homeostasis in a bioengineered vascular graft.

5 Conclusions

The current work demonstrates endothelial alignment in response to aligned nanofibrillar collagen. This form of collagen modulates EC organization and migration, and supports EC phenotype in the form of a vascular graft. Ongoing pre-clinical studies will determine the promise of this material in maintaining endothelial homeostasis, and preventing processes involved in vascular disease.

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Executive Summary

Introduction

- The morphology of vascular endothelial cells (ECs) is linked to their biological responsiveness. It is worthwhile to understand how a patterned matrix can fundamentally control the morphology (and the function) of ECs.
- The purpose of this study was to develop a straightforward technique to fabricate anisotropic nanofibrillar collagen, and to assess the effect of matrix anisotropy on EC organization and migration, for applications in vascular tissue engineering.

Materials & Methods

- An extrusion flow processing technique is presented that aligns molecules of collagen and induces fibrillogenesis to generate anisotropic nanofibrillar collagen.
- Primary human dermal microvascular ECs were cultured on aligned or on randomly oriented collagen and their cytoskeletal organization and alignment were characterized by staining for the actin filaments and paxillin.
- Time-lapse microscopy was used to observe the migration of ECs on the nanofibrillar collagen for 24 hours. The cellular protrusions were manually tracked to quantify cell migration directionality and velocity.

Results

- Extrusion flow processing is a reproducible and facile method for fabricating aligned nanofibrillar collagen scaffolds
- Anisotropic collagen nanofibrils modulated EC morphology by organizing the actin cytoskeleton, cell shape, and focal adhesions.
- EC migration speed and direction is also influenced by the anisotropy of collagen nanofibrils. The cells on aligned collagen probed with more directionality and with faster velocity along the collagen fibril direction.
- The flow technique can be adapted to fabricate a vascular graft of collagen fibrils, with physiologically relevant elastic modulus and burst pressure. The aligned vascular conduit could support endothelial attachment, phenotype, and proliferation.

Conclusion

- The current work demonstrates endothelial alignment in response to anisotropic nanofibrillar collagen. This form of collagen modulates EC morphology and migration, and supports EC phenotype in the form of a vascular graft.

Fig. 1. Fabrication of aligned collagen nanofibrillar strips by extrusion flow processing
 A. Schematic of flow technique (side view) to produce aligned collagen strips. The hydrodynamics of the flow technique align the rod-shaped molecules of collagen along the direction of flow. Top view of strips of collagen (gray) adhered on glass slide. B. SEM of randomly oriented and aligned matrices of collagen nanofibrils. Inset depicts frequency plots using 2D fast Fourier transform (FFT) analysis. C. 2D FFT alignment plots. Arrow denotes direction of collagen alignment. Scale bar: 200 nm.

Fig. 2. Aligned matrix of collagen nanofibrils regulates endothelial cell (EC) organization
A–B. ECs align along the collagen fibril direction when grown on the aligned collagen, as demonstrated by phalloidin staining for F-actin. C. Quantification of the absolute average degree of alignment, which is quantified as the angle formed by the direction of the cell, relative to the direction of the collagen nanofibrils. D. Frequency distribution plot of the degree of alignment. E. 2D FFT frequency plot and quantification of 2D FFT frequency plot. F. Immunofluorescence staining of F-actin and paxillin focal adhesion proteins for a single cell. Arrow denotes direction of collagen alignment. * $P < 0.05$. Scale bar: A–B) 100 μm , F) 25 μm .

Fig. 3. Aligned nanofibrillar collagen directs EC migration

A. Time-lapse images show the elongation of a cell along the direction of the aligned collagen fibrils. B. Tracking of cell's protrusion movement over 24 h as depicted by vector plots and Rose diagrams (inset). C. Quantification of mean squared displacement (MSD) in the *X* (parallel to aligned collagen fibrils) and *Y* (perpendicular to aligned collagen fibrils) axes as a function of time. D. Velocity of cell protrusion along the *X* and *Y* directions. Arrow denotes direction of collagen alignment. * $P < 0.05$, *** $P < 0.001$.

Fig. 4. Characterization of the aligned collagen vascular grafts

A. Flow schematic for fabricating a vascular graft. The inner mandrel extrudes the collagen through an annulus, aligning the collagen molecules. B. Photo micrograph of aligned collagen conduit. C. Mechanical characterization of true stress as a function of strain. D. Burst pressure measurement.

Fig. 5. Endothelial response on the anisotropic nanofibrillar vascular grafts
A–B. SEM of ECs on aligned vascular grafts. C. Confocal microscopy of CD31 and Ki67 expression on vascular grafts. Arrow denotes direction of collagen alignment. Scale bar: A) 50 μm , B) 20 μm , C) 75 μm .