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Surface Projections of Titanium Substrates Increase Antithrombotic Endothelial Function in Response to Shear Stress

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Abstract

Despite the therapeutic benefits of both mechanical circulatory assist devices and nitinol stents with titanium (Ti) outer surfaces, problems remain with thrombosis at the blood-contacting surface. Covering these surfaces with a layer of endothelium would mimic the native lining of the cardiovascular system, potentially decreasing thrombotic complications. Since surface topography is known to affect the phenotype of a seeded cell layer and since stents and ventricular assist devices exhibit surface protrusions, we tested the hypothesis that endothelial cells (ECs) have altered function on Ti surfaces with protrusions of 1.25, 3, and 5 μm height, compared to smooth Ti surfaces. ECs and nuclei were more aligned and ECs were more elongated on all patterned surfaces. Cell area was reduced on the 3 and 5 μm features. Expression of eNOS and COX2 was not altered by patterned surfaces, but expression of KLF-2 was higher on 1.25 and 5 μm features. Nitric oxide production following exposure to flow was higher on the 5 μm features. These results show that some antithrombotic functions of ECs are significantly enhanced for ECs cultured on surface protrusions, and no functions are diminished, informing the future design of implant surfaces for endothelialization.

Keywords

titanium; endothelial cells; topography; vascular stents; shear stress

1. Introduction

Blood-contacting medical devices such as nitinol stents and mechanical circulatory assist devices (MCADs) are composed of titanium oxide at the outer surface. Covering the blood-contacting surfaces of these devices with a layer of functional endothelium could ameliorate issues with thrombogenicity of the material under certain flow conditions.¹ The effect of surface topography on a seeded endothelial cell (EC) layer is of particular interest because existing titanium (Ti) implants have rougher surfaces than glass slides or tissue culture plastic often used in biomedical research. We have previously seeded ECs onto Ti implants and found that these cells function as well as they do on tissue culture plastic.² Surface topography is of interest in its ability to support an endothelial cell layer directly seeded onto an implant surface prior to implantation, as we have previously proposed.³ Additionally, the topography of cardiovascular implant surfaces can encourage endothelialization from an adjacent vessel wall, after implantation.^{4,5} Accelerated endothelialization of stents is associated with reduced thrombosis and intimal thickening.^{6,7} Prospective design of cardiovascular implant surfaces for enhanced endothelialization before implantation or *in*

situ after implantation will benefit from a greater knowledge of how surface features affect an adherent endothelial layer.

Surface topographic features, such as grooves, unordered roughness, and posts or protrusions, influence ECs in contact with those surfaces. ECs align and migrate along the length of grooves with widths of 300 nm – 10 μ m and depths of 300 nm - 5 μ m, without the application of fluid shear stress.^{5,8–10} Liliensiek et al. showed that flow primarily determined orientation of ECs adherent on closely spaced (400 – 800 nm) polyurethane ridges, but that surface topography had a greater influence on orientation for larger feature spaces (2000 – 4000 nm).¹⁰ When fluid shear stress is applied, endothelial cells align and elongate in the direction of flow, which is enhanced if the surface is endowed with long grooves or ridges along the same direction.^{5,11} Likewise, endothelial nuclei align along the direction of grooves, when flow is applied in the same direction as the surface grooves.¹² Furthermore, grooves enhance surface retention of ECs exposed to flow.^{13,14} When flow is directed perpendicular to long grooves, a more limited reorientation of cells and endothelial nuclei in the direction of flow is observed,^{5,12} dependent on factors such as the feature size and magnitude of applied shear stress. Nuclear size and volume have also been found to increase on more widely spaced grooves.¹⁵ EC proliferation increased as groove size decreased from 750 μ m to 0.1 μ m.⁸

In contrast to ordered grooves, unordered surface roughness of varying magnitude may better replicate the structure of the extracellular matrix to which ECs normally adhere. Surfaces, with random topography, have been produced by ion beam etching,^{16,17} electron beam deposition,¹⁸ or sand blasting.¹⁹ EC proliferation and adhesion were reduced on surfaces of random roughness on the order of 300 – 1000 nm root mean squared (RMS),²⁰ but surfaces with randomly generated features in the range of 50 – 100 nm dimension caused enhanced EC proliferation and ECM production.¹⁶ A study by Lipski et al. showed that surfaces with nano-spheroids (RMS 2.44 – 69.38 nm) caused increased F-actin alignment in subconfluent ECs as roughness increased, compared to glass controls.²¹ EC migration was increased on nanometer- and submicron-scale surfaces roughened with electron beam deposition.²² The submicron scale roughness also reduced platelet adhesion. These surfaces preferentially promoted EC growth over smooth muscle cell (SMC) growth, which could be advantageous for reduction of SMC-related neointimal hyperplasia. Correspondingly, roughened stent surfaces (RMS 0.09 – 0.21 μ m) have been implicated in reduced angiographic restenosis *in vivo*.¹⁹

Although long grooves have been studied extensively, stents and MCADs exhibit surface protrusions. Some ordered post-like or bump-like features with endothelial monolayers have previously been studied. Micropillars of polydimethylsiloxane (PDMS) 1 – 5.6 μ m diameter with spacing varying from 0.6 – 15 μ m have been studied as an EC substrate, and smaller posts were found to support higher cell density in static culture.²³ The same effects were noted for ridges of similar spacing. Micropillars of tissue culture polystyrene 3 μ m diameter and 0.5 μ m height were studied in conjunction with application of flow for 1 hour¹¹. In these short-term experiments, retention, rather than reorganization with flow, was studied and found to be reduced on the pillar surfaces compared to planar or ridged surfaces. Surface protrusions on titanium surfaces have not been investigated for their ability to support endothelialization.

Toward our goal of seeding cardiovascular devices with endothelium prior to implantation, we investigated titanium surfaces with protrusions of height 1.5 – 5 μ m and base dimensions of 3.5 – 12.5 μ m, and their effect on an endothelial layer exposed to flow. We tested the hypothesis that following exposure to flow endothelial cells have altered function on Ti

surfaces with micron-size protrusions, compared to smoother Ti-covered glass surfaces – as evaluated by EC gene expression, spreading, elongation/roundness, and alignment.

2. Materials & Methods

EPC isolation and culture

ECs were isolated from human umbilical cord blood, as we have done previously.^{24,25} Umbilical cord blood samples were obtained from the Carolina Cord Blood Bank at Duke University per protocols approved by the Duke Institutional Review Board.

ECs were cultured using standard practices in EBM-2 base medium + EGM-2 SingleQuots (Lonza).²⁴ Cells were plated at passages 5 – 9 for flow studies. At 24 hours prior to the onset of flow, 1.25×10^6 cells were plated per slide, with each slide for flow being matched to a static control at the time of plating. Cells were confluent at the time of experiments.

For flow cytometry, ECs were incubated with 0.025% trypsin (Lonza) for 3 minutes at 37°C and the reaction was neutralized with Trypsin Neutralizing Solution (Lonza). Cells were incubated at room temperature with FITC-conjugated antibodies to EC markers CD31, CD105, and CD146, as well as monocyte markers CD14 and CD45 (Serotec) for 30 minutes. Cells were rinsed and pelleted and fixed in 3.7% paraformaldehyde. Isotype controls (mouse IgG1, mouse IgG2a, Serotec) were used for each condition as appropriate. Approximately 9,000 cells were counted for each antibody on a FACStar flow cytometry machine.

Surface fabrication

Slides with evenly spaced pyramidal protrusions of 5 μm , 3 μm , and 1.5 μm height were designed for fabrication on 6-inch silicon wafers by photolithography. Space between each base was designed to be consistent, approximately 3 μm . A silicon oxide layer of 5 μm thickness was first deposited, following by masking with photoresist and etching. Wafers were diced before etching into 1 inch by 3 inch dimensions, with an identical footprint as standard microscope slides. A layer of Ti of 99.999% purity, 100 nm thick, was sputter-coated on the patterned silicon slides and on piranha-cleaned standard glass slides (Fisher Scientific), which served as a planar control. Ti was sputter-coated at a deposition rate of 1.7 Å/s using a Kurt J Lesker PVD 75 DC sputter system. Slides were rotated continuously during the deposition process.

Surface characterization

Surface roughness and peak-valley height were measured by optical profilometry / 3D surface metrology system (Zygo NewView 5000).³ Features were examined in detail by scanning electron microscopy (SEM), and slides were also examined at low magnification by SEM for measurement of any defect areas in which features were missing or misshapen. Spacing and lateral dimensions of protrusions were estimated by measuring distances in high-resolution SEM images with ImageJ software.

Flow experiments

Ti-coated patterned slides were exposed to steady laminar flow in a parallel plate flow chamber for 48 hours to simulate physiological shear stress as we have done previously.^{2,24} The applied shear stress of 15 dyne/cm² was achieved by applying a flow rate in the range of 60 – 100 mL/min. The flow rate was dependent on the measured flow chamber height according to equation (1), where Q is flow rate, τ is the shear stress acting on the endothelial layer, w is chamber width, h is chamber height, and μ is viscosity of the flow medium.

$$Q=(TWh^2)/(6\mu) \quad (1)$$

DMEM-F12 (Lonza) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Sigma) was used as the medium for the flow experiments and static controls. Following 48 hours exposure to either flow or static conditions, the slide was divided with Teflon tape into unequal portions; cells were trypsinized and collected from the larger part of the slide for RNA extraction, and the cells on the smaller part of the slide were rinsed and fixed for subsequent immunostaining.

Nitric oxide quantification

To quantify nitric oxide (NO) production by ECs, we directly measured the primary oxidation product nitrite (NO_2^-) in 150 μL medium samples collected from the flow circuit reservoir and static control at 0 h and 48 h in 12 experiments. Nitrite is a commonly used surrogate of NO availability²⁶ and has been shown to reflect changes in NO bioavailability following physical stimuli more effectively than measuring nitrate (NO_3^-) or the combined quantity of nitrate and nitrite (NO_x).²⁷ Samples were frozen at -80°C until further analysis. The concentration of NO_2^- was measured by chemiluminescence with an Ionics/Sievers Nitric Oxide Analyzer (NOA 280, Sievers Instruments, Boulder, CO) as described previously.²⁶ The total amount of nitrite produced was calculated as the product of concentration produced times the total volume of the circuit (100 mL) or static control (10 mL) while adjusting for volume lost from taking samples.

Immunohistochemistry, fluorescent microscopy, & image analysis

EC-coated Ti slides for imaging were fixed in 3.7% buffered formaldehyde (Ricca Chemical) and permeabilized with 0.1% TritonX-1000 (Sigma-Aldrich). Cells were blocked in 10% goat serum and incubated with mouse anti-human CD31 antibody (Invitrogen) diluted 1:100. Following, cells were incubated with the secondary antibody goat anti-mouse IgG AlexaFluor 488 (Invitrogen), diluted 1:500. Nuclei were counterstained with Hoechst 34580 (Invitrogen), diluted 1:1000. Actin was stained on selected samples with rhodamine phalloidin, diluted 1:100. Fluorescent microscopy was performed with an inverted Nikon Eclipse TE2000-U microscope with a monochrome digital camera. Area and angles were determined for 100 cells per sample and 25 nuclei per sample using ImageJ software. Cell and nuclear roundness were calculated according to equation (2).² Roundness by this equation ranges from 0, for a straight line, to 1, for a circle. Values closer to 0 represent more elongated cells.

$$\text{Roundness}=4\pi \bullet \text{Area}/(\text{Perimeter}^2) \quad (2)$$

Cell and nuclear angles were determined in the ImageJ software, as the angle of each shape's major axis, transformed to values in the first quadrant, between 0 and 90 degrees. To calculate nuclear intensity of Hoechst 34580 staining, mean gray values were collected for 25 nuclei and 25 separate areas of background in a single monochrome blue-channel image, for flow samples only. The mean gray value for each nucleus was divided by the average background gray value for each image, and averaged for each experiment. Relative nuclear intensity is reported as that ratio. For three-dimensional reconstruction, ECs cultured in static conditions on each surface were stained with CellTracker Orange and Hoechst 34580, then imaged in three dimensions with a Zeiss 780 confocal upright microscope. Zen 2010 software was used to create orthographic projections of the three-dimensional reconstruction.

Quantitative RT-PCR

ECs were characterized for expression of Krüppel-like factor 2 (KLF-2), endothelial nitric oxide synthase (eNOS), and cyclooxygenase-2 (COX2). Total RNA was isolated for gene expression analysis (Aurum Total RNA Mini Kit, Bio-Rad). cDNA was synthesized using the iScript kit (Bio-Rad). Quantitative RT-PCR was performed (iQ SYBR Green Supermix, Bio-Rad) as described previously^{24,28} with the Bio-Rad MyIQ iCycler to evaluate expression of antithrombotic genes eNOS, COX2, and transcription factor KLF-2. The housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) served as endogenous control for target gene expression, and cells grown in culture flasks under normal conditions provided reference RNA. The $2^{-\Delta\Delta CT}$ method was used to determine the relative gene expression, modified for primer efficiency.²⁹ Efficiency of each primer was measured and used to find $\Delta\Delta CT$.³⁰ Measured primer efficiencies were 86% for both KLF-2 and GAPDH, and 100% for both eNOS and COX2. The forward and reverse primers used, selected based on the gene sequence of interest, were respectively for each gene:

- a. endothelial nitric oxide synthase (eNOS): 5'-GTG ATG GCG AAG CGA GTG AAG-3' and 5'-CCG AGC CCG AAC ACA CAG AAC 3';
- b. cyclooxygenase-2 (COX2): 5'-TGA GCA TGT ACG GTT TGC TG-3' and 5'-TGC TTG TCT GGA ACA ACT GC-3';
- c. Krüppel-like factor-2 (KLF-2): 5'-GCA CGC ACA CAG GTC AGA AG-3' and 5'-ACC AGT CAC AGT TTG GGA GGG-3';
- d. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-GAC CCC TTC ATT GAC CTC AA-3' and 5'-CAT GGA CTG TGG TCA TGA GC-3'

Melting curves were obtained after each reaction to verify that only the target cDNA was amplified.

Statistical methods

EC angle, roundness, and area were analyzed using ImageJ software in 100 cells per sample. For each condition, a total of $n = 3$ replicates was examined. Endothelial cell nucleus angle was analyzed in 25 cells per sample and averaged for each experiment, for a total of $n = 3$ experiments per condition. Values were compared with two-factor ANOVA and Bonferroni post-hoc test.³¹ PCR amplification was performed in triplicate, and threshold cycles were averaged to obtain the value used in the analysis. Nitric oxide production, nucleus staining intensity, and the gene expression fold-changes were compared with one-way ANOVA and Dunnett's post-hoc test.³² All values are reported as means $\pm$ standard error of the mean. Values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were performed using GraphPad Prism 5 and JMP 8 software.

3. Results

EPC Isolation and characterization

ECs derived from cord blood formed confluent monolayers on all surfaces and exhibited characteristic endothelial cell cobblestone morphology. Flow cytometry confirmed that the cell population was positive for endothelial markers CD31, CD105, and CD146, previously found to be positive in blood-derived endothelial progenitor cells and other endothelial

cells.³³ Cells were negative for monocyte and leukocyte markers CD14 and CD45, respectively, typical of ECs derived from late outgrowth blood-derived EPCs.³³

Surface roughness / surface characterization

SEM analysis revealed the patterned slides to be uniform, with any defects affecting < 1% of total area. Surface protrusions were found to be approximately of the same dimensions designed (Fig. 1). The base dimensions of protrusions were approximately 12.5 μm , 7 μm , and 3.5 μm , respectively, but distance between bases was designed to be consistent and was found to range only from 4 – 5 μm in each feature size. Distance between peaks of the features increased accordingly with increasing feature size. Peak-valley height for the largest, mid-sized, and smallest features were 4855 nm $\pm$ 40 nm, 3033 nm $\pm$ 155 nm, and 1257 nm $\pm$ 179 nm, respectively. Values reported as mean $\pm$ SE (n = 3 samples per condition).

Based on the reported values of peak-valley height, the local shear stress, designated τ , at the top of each protrusion was calculated as a proportion of the wall shear stress for undisturbed flow using the following equation reported by Barbee et al.³⁴

$$\tau/\tau_w = 1 + \kappa(h - h_{\text{avg}}) \quad (3)$$

where τ_w is the wall shear stress in the absence of the protrusion and $h - h_{\text{avg}}$ is the variation in surface height from the mean of a single protrusion. The constant κ is used as 0.120 μm^{-1} for cells prior to exposure to flow.³⁴ The local shear stress over the protrusion was calculated to be increased over the average level by 7.5%, 18%, and 29%, for the features measured to be approximately 1.25 μm , 3 μm , and 5 μm height, respectively. Because protrusions were designed to have equal spacing between the bases and bases of differing size, the spaces between peaks varied, from approximately 7.5 μm to 18 μm . The lateral footprint and surface area of each protrusion was different, with lateral footprints ranging from approximately 13 to 160 μm^2 , and surface areas ranging from approximately 16 to 200 μm^2 .

Cell alignment, roundness, and area

Following 48 hours exposure to physiological shear stress, cells on all surfaces aligned in the direction of flow and cells on larger protrusions appeared smaller (Fig. 2). ECs on all surfaces aligned significantly with flow compared with static controls ($p < 0.0001$, $n = 3$) [Fig. 3(A)]. Furthermore, ECs on all patterned surfaces aligned with flow to a greater extent than the ECs on planar controls, as denoted by a lower average angle ($p < 0.05$, $n = 3$), although no significant difference was seen among the various feature sizes [Fig. 3(A)]. Under static conditions, ECs on all surfaces demonstrated a random overall orientation, represented by an average angle of approximately 45°. Likewise, following 48 hours exposure to physiological shear stress, nuclei on all surfaces significantly aligned in the direction of flow compared to static controls ($p < 0.0001$, $n = 3$) [Fig. 3(B)]. EC nuclei on all patterned surfaces aligned with flow significantly more than the nuclei on planar controls, as denoted by a lower average angle ($p < 0.05$, $n = 3$), although no significant differences were seen in angles among the patterned surfaces. Nuclei on all surface types demonstrated a random overall orientation under static conditions, as with the cell orientation. Neither nuclear area nor nuclear roundness significantly changed between static and flow conditions, or among surface types (data not shown). Nuclear roundness ranged from 0.82 to 0.90.

Patterned surfaces caused enhanced elongation of ECs with flow, as evidenced by a significant decrease in calculated cell roundness on the patterned surfaces in the flow condition compared to the planar controls ($p < 0.05$, $n = 3$) [Fig. 3(C)]. Cell area was

significantly reduced on the largest and mid-sized features when compared to the smallest features and planar controls ($p < 0.05$, 3 μm features and $p < 0.01$, 5 μm features; $n = 3$) [Fig. 3(D)]. Staining intensity of nuclei with Hoechst 34580 following 48 hours physiological flow was assessed as a measure of chromatin availability,³⁵ and average nuclear intensity was calculated for each surface. Staining intensity of nuclei was not significantly different on any surface but had a slightly increasing trend with surface feature size (Fig. 4).

Impact of protrusions on cell topography and shear stress

We examined 3-D reconstructions of the cell layer on each type of protrusion to evaluate to assess the effect of the protrusion on basal surface and the EC surface exposed to flow. For all protrusion sizes, the cell's lower surface conformed to the protrusions and did not skip over the tops of the bumps [Fig. 5]. The 1.25 μm and 3 μm protrusions did not appear to alter the upper surface of the ECs. For these protrusion sizes, the nucleus had a greater effect on the upper surface contour of the cell than did the protrusions. In contrast, the 5 μm protrusions were largest compared to the ECs, and defined peaks could be seen at the cells' upper surface, corresponding to the topography of the patterned substrate [Fig. 5]. For this protrusion size, the topography had a greater effect on the cell contours compared to the nuclei, which were often positioned between the protrusions.

The protrusions affected the nuclei positioning over the ordered array of protrusions. Because protrusions were designed to have equal spacing between the bases and bases of differing size, the spaces between peaks varied, from approximately 7.5 μm to 18 μm . The lateral footprint and surface area of each protrusion was different, with lateral footprints ranging from approximately 13 to 160 μm^2 , and surface areas ranging from approximately 16 to 200 μm^2 . Therefore, for 1.25 μm tall protrusions, each cell nucleus appears to span multiple protrusions (Figure 2B). For 3 μm tall protrusions, many cell nuclei sit entirely in the depressions between protrusions, while others span approximately one protrusion (Figure 2C). For 5 μm tall protrusions, most cell nuclei sit in the depressions between the pyramid peaks (Figures 2D and 5A). In a minority of observed nuclei, nuclei span the peaks of the pyramidal shapes. As a result, cells with nuclei on top of protrusions will experience a greater increase in shear stress over parts of the cell than those cells with nuclei sitting in the depressions between protrusions. Nuclei on all surfaces significantly aligned with flow, as others have previously reported,^{5,12} and presence of the surface protrusions enhanced nuclear alignment.

Based on these observations, the local shear stress, designated τ , at the top of each protrusion was calculated as a proportion of the wall shear stress for undisturbed flow using the following equation reported by Barbee et al.³⁴

$$\tau/\tau_w = 1 + \kappa(h - h_{\text{avg}}) \quad (3)$$

where τ_w is the wall shear stress in the absence of the protrusion and $h - h_{\text{avg}}$ is the variation in surface height from the mean of a single protrusion. The constant κ is used as 0.120 μm^{-1} for cells prior to exposure to flow.³⁴ The local shear stress over the bare protrusions was calculated to be increased over the average level by 7.5%, 18%, and 29%, for the features measured to be approximately 1.25 μm , 3 μm , and 5 μm height, respectively. However, we found with 3-D confocal reconstruction that only the 3 μm and 5 μm protrusions appear to have an effect on the apical surface, and therefore 18% and 29% are a maximum expected increase in apical shear stress on those surfaces. In contrast, the 1.25 μm surface is not expected to have an increase in apical shear stress. Neither the 1.25 μm nor 3 μm surfaces appear to push up the cell nucleus significantly. The EC apical surface on the 3 μm surfaces

are primarily affected in thinner regions away from the nucleus. With the 5 μm protrusions, nuclei primarily settle between protrusion peaks, meaning an additive effect of the protrusion and nucleus height is not expected in most cells.

Nitric oxide production

Nitric oxide (NO) levels, evaluated by directly measuring nitrites, showed that there was no deleterious effect of the surface features on EC production of NO, an antithrombogenic molecule. After exposure of ECs to flow for 48 hours, nitrite production was significantly higher in all flow conditions than in randomly selected static samples when normalized for circuit volume. Static samples had total nitrite values ranging from approximately 0.75 – 3.15 nmol. Nitrite production was positive in all cases after subtraction of the time zero values. Nitrites appeared to have an increasing trend with surface feature size (Fig. 6), but only the production on the 5 μm height surfaces was significantly greater than control (* $p < 0.05$, $n = 3$).

Gene expression

Gene expression was measured for ECs under static conditions and after exposure to flow for 48 hours. Gene expression under static conditions did not vary among the different surfaces with respect to reference RNA. Expression of eNOS and COX2 in the flow condition was not significantly changed on different surface topographies [Fig. 7(A,C)]. Expression of KLF-2 with flow was significantly higher on the small (1.25 μm) and large (5 μm) features than on the control ($p < 0.05$, one-way ANOVA and Dunnett's post-hoc test) [Fig. 7(B)].

4. Discussion

Surface protrusions on Ti substrates altered the response of ECs to physiological flow. Following exposure to 15 dyne/cm² for 48 hours, the presence of surface protrusions in this study increased both the elongation of the cells and reduced the cell angle, representing increased alignment with flow. The cell area on the 3 μm and 5 μm surfaces was reduced for both flow and static conditions; this finding may reflect an inability of ECs to spread over the larger protrusions, or a higher initial attachment and consequent tighter spacing. Expression of KLF-2 was increased in 5 μm and 1.25 μm protrusions compared to control, but was not changed on 3 μm protrusions. Both eNOS and COX2 expression were unchanged on the patterned surfaces compared to controls. Importantly, all genes investigated were elevated in the flow condition with respect to static values, on every surface type. Nitrite concentration as a measure of NO in flow samples showed an increasing trend with surface protrusion size and was significantly greater on 5 μm protrusions than the control surface.

Increases in some of these typical EC responses to flow suggest a beneficial effect of the patterned surfaces on healthy EC function and a similarity to *in vivo* unperturbed flow.³⁶ Although long surface channels or grooves have been studied more extensively, this study represents the first examination of the combined effect of physiological flow and EC culture on a surface with periodic “bump-like” protrusions.

Most previous studies of surface topography and endothelium have focused on longitudinal grooves and their impact on flow. Other studies have investigated micropillar surface protrusions, but only in static culture²³ or under short (1 hour) flow conditions.¹¹ In a study of ECs on surfaces with ordered holes, Morgan et al. found that surfaces with holes of various dimensions up to 4 μm pitch did not significantly change the extent of EC or nucleus realignment to flow.¹² They and others have found that grooved surfaces, in contrast,

enhance alignment along the direction of flow or hinder alignment when perpendicular to flow. Morgan et al. concluded that topographical features without an overarching directional orientation were unable to elicit the synergistic or antagonistic effects on EC alignment with flow. Protrusions examined in this study did not provide a continuous longitudinal orientation, but were periodic in nature. Despite this, our arrayed protrusions increased the degree of cell and nuclear alignment with flow in contrast to the statement made by Morgan et al. The nature of our features as protrusions rather than holes and the corresponding increase in local shear stress can account for this difference in EC response. Further, for small holes, the cells may cover the hole without forming a depression on the cell surface. Previous studies of surface topography have not focused on gene expression of ECs on different surfaces, which is an addition of the current study.

Increases in endothelial cell and nucleus alignment, cell elongation, and nitric oxide production on some or all patterned surfaces can be explained by increased local shear stress over the surface protrusions. The increase at the apical surface ranged from an estimated 18% in the mid-sized features to 29% in the largest features studied, with no effect observed on the apical surface with the smallest features. With surface protrusions and nuclei both contributing to deviations from a perfectly planar surface, flow recirculation can occur at the points of disturbance. In a study by Pozrikidis, flow streamlines over hemispherical protrusions of different aspect ratios are presented.³⁷ In a full hemisphere, stagnation points are mathematically predicted to occur at the protrusion base. In contrast, when the aspect ratio of the semi-spheroidal height to base diameter is 0.5, no stagnation or recirculation is observed. We do not expect flow reversal for the conditions we studied because the ratio of height to base length of the pyramidal protrusions is 0.35–0.4, representing shallower protrusions than those described which showed no recirculation. Further, 3-D reconstructions of ECs on these surfaces in static culture indicate that the cells' upper surface was smoother than pyramids in every case. Only for the 5 μm and 3 μm projections did the projection alter the topography at the upper surface.

KLF-2 is a transcription factor whose upregulation has previously been shown to increase expression of eNOS and other shear sensitive anti-inflammatory and antithrombotic genes in endothelial cells.³⁸ In our study, the surfaces which exhibited increased KLF-2 expression were those with 5 μm and 1.25 μm protrusions. However, these did not also show corresponding increases in eNOS expression, suggesting competitive interactions or influence of other factors affecting eNOS expression.³⁹ The gene expression of KLF-2 did not show a monotonic trend, indicating other factors influencing expression of that gene.

The intensity of nuclear staining with DNA-specific dyes is related to chromatin structure, which is roughly correlated with transcriptional activity.³⁵ Therefore, changes in nucleus intensity could represent global changes in overall gene expression, but no significant differences were found. This result suggests the effect of surface patterning was not sufficient to cause a global change in gene expression, although there are likely to be changes in specific genes due to flow and shear stress over the protrusions.

On flat surfaces, the genes studied (eNOS, COX2, KLF-2) are upregulated with shear stress, consistent with our findings on the patterned surfaces compared to static controls.^{24,40} The presence of surface protrusions did not inhibit expression of the antithrombotic genes investigated compared with the control surface, and did not prevent their upregulation with flow, supporting that there is no negative effect of the patterned titanium surfaces on the confluent endothelial layer.

Although expression of eNOS mRNA was not found to significantly change with protrusion size, nitrite concentration as a measure of NO showed an increasing trend with surface

protrusion size and was significantly greater on 5 μm protrusions than the control surface. Factors other than eNOS gene expression alone can regulate the activity of the eNOS protein,⁴¹ accounting for the change in NO not matching the gene expression levels of eNOS. For example, phosphorylation of eNOS is increased with increased shear stress,^{42,43} and increased phosphorylation increases the activity of the eNOS enzyme.^{44,45} In this way, the increased shear stress experienced by ECs on the largest protrusions could lead to more NO production in the absence of increased mRNA levels.

Since the apical cell surface contour depends upon the dimensions of the protrusions and the density of the protrusions changed with size of the protrusion, then the mechanotransduction pathway depends upon the size of the protrusion. All of the protrusions studied deform the basal surface and increase the basal surface area. Only the 5 μm protrusions led to a significant alteration of the apical surface of the ECs; surface changes with the 3 μm protrusions appeared minor. As noted, these protrusions would increase the local shear stress on the endothelial cells at the apex of the protrusions. Following application of fluid shear stress over the endothelium, protrusion of the cell nucleus causes stress concentration upon the focal adhesions beneath the nucleus.⁴⁶ Consequently, the 2–4 protrusions on cells grown on the 5 μm protrusions would increase the regions at which stress concentration occurs, amplifying the signal produced by the fluid shear stress. The 5 μm protrusions would be more likely to exhibit sensitivity to shear stress as evidenced by NO release and KLF-2 expression [Figures 6 and 7]. If correct, then activation of the ERK5 pathway would be greater for cells on the 5 μm protrusions than on the other protrusions.⁴⁷ That the ECs exhibited similar shape changes on all three types of protrusions after exposure to flow suggests that these shape changes are driven by the deformation of the basal surface. The protrusions do not retard the alignment of the cells following exposure to flow but do help to align the cells and nucleus and may be driven by cell-substrate interactions. Since Jun NH2-terminal kinase (JNK) is activated by flow and regulates endothelial cell alignment under flow,⁴⁸ this pathway may be altered by the presence of the protrusions. Investigation of signaling pathways activated by flow for endothelial cells on protrusions could provide important insights into the regulation of endothelial function on vascular implants and engineered constructs.

5. Conclusions

These studies show that surface roughness of Ti substrates alters typical responses to flow exhibited by ECs, including cell and nuclear alignment, cell area, and cell elongation/roundness. Increased elongation, alignment, nitric oxide production, and expression of KLF-2 correspond to higher local shear stress. Surfaces with protrusions increased several antithrombotic EC functions, suggesting that surface variations and protrusions of the scale studied can have a positive effect on a seeded EC layer. The protrusions had no deleterious effects on EC function. These studies provide proof of concept for application of EC seeding to Ti implants of various surface roughnesses and can serve to inform implant design for endothelial coverage.

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Figure 1.

SEM image of (A) largest size, (B) mid-sized, and (C) smallest size features. (D–G) Optical profilometry surface scans. (D) Largest feature size, peak- valley height = $4855 \text{ nm} \pm 40 \text{ nm}$, scan area $140 \times 110 \text{ } \mu\text{m}$. (E) Mid-sized features, peak-valley height = $3033 \text{ nm} \pm 155 \text{ nm}$, scan area $140 \times 110 \text{ } \mu\text{m}$. (F) Smallest feature size, peak-valley height = $1257 \text{ nm} \pm 179 \text{ nm}$, scan area $70 \times 50 \text{ } \mu\text{m}$. (G) Planar Ti-covered glass control, roughness average (R_a) = $0.73 \text{ nm} \pm 0.07 \text{ nm}$, scan area $720 \times 540 \text{ } \mu\text{m}$. Note: Values reported as mean $\pm$ SE ($n = 3$).

Figure 2.

ECs following 48 hours physiological flow on (A) Planar control surface, (B) 1.25 μm protrusions, (C) 3 μm protrusions, and (D) 5 μm protrusions. Red: Actin (rhodamine phalloidin); Green: PECAM-1 (CD31); Blue: Nuclei (Hoechst 34580). Scale bars represent 100 μm . Protrusions can be seen underneath cells in (B–D). Direction of flow is parallel to horizontal in every image.

Figure 3.

(A) Surface protrusions caused a significant decrease in the angle of cells in the flow condition compared to control (* $p < 0.05$), although no significant difference was observed among the feature sizes. (B) Surface protrusions caused a significant decrease in the angle of cell nuclei in the flow condition compared to control (* $p < 0.05$), although no significant difference was observed among the feature sizes. (C) Surface protrusions caused a significant decrease the roundness of ECs in the flow condition compared to control (* $p < 0.05$), although no significant trend was observed due to the feature size. (D) Cell area was significantly reduced in both the flow and static condition on the 3 μm (* $p < 0.05$) and 5 μm features (** $p < 0.01$), compared to the 1.25 μm and control surfaces. For (A–D), $n = 3$ in each condition.

Figure 4.

Nucleus staining intensity in cells exposed to flow for 48 hours did not significantly change with surface type, but had a slightly increasing trend with surface feature size.

Figure 5.

Orthographic projections of the three-dimensional contours of ECs cultured on features with heights of 5 μm (A), 3 μm (B) and 1.25 μm (C). Orthographic projections were obtained via confocal microscopy on fixed cells stained with CellTracker Orange (red) and Hoechst 34580 (blue). Sections show that on 5 μm projections EC upper surface is distorted by underlying surface topography (A), but that EC upper surface is not noticeably influenced by underlying surface topography on 3 μm and 1.25 μm projections (B–C). Sections of ECs near the surface confirmed that the bottom surface of cells conform around protrusions of every size studied (Data not shown). Scale bars represent 10 μm .

Figure 6.
Nitrite production after 48 hours flow on all surfaces. (* $p < 0.05$)

Figure 7.

Effect of feature size on gene expression relative to static conditions. (A) Expression of eNOS did not significantly vary with surface feature size. (B) KLF-2 expression of ECs on 1.25 μm and 5 μm tall protrusions was significantly greater than control (* $p < 0.05$, one-way ANOVA and Tukey post hoc test). (C) COX2 expression with flow did not significantly change with surface feature size.