RATIONALLY-DESIGNED SURFACE NANOPATTERNING: A POTENTIALLY NEW MEANS FOR ENHACING SAFETY & EFFICACY OF VASCULAR STENTS

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ABSTRACT

This paper presents preliminary data supporting the feasibility of an innovative new therapeutic strategy that seeks to address the limitations of current drug-eluting stents via rational design of stent surface topography at the nanoscale. Using recently-developed anisotropic metal micromachining techniques, we have created bulk metal substrate surfaces patterned with arrays of micrometer to sub-micrometer scale parallel grooves. The rationale for this pattern geometry is based upon the presumption that such features will facilitate healing by accelerating reestablishment of the endothelium, the native lining of the vessel wall. Results from our preliminary *in vitro* studies show that these surfaces accelerate endothelialization, promote more native cellular morphology, and significantly enhance competitive adhesion of endothelial cells over vascular smooth muscle cells. As such, these data suggest that rationally-designed nanopatterned surfaces may provide a robust new means for enhancing efficacy and safety of vascular stents, possibly without the need for drug-elution.

INTRODUCTION

Coronary heart disease (CHD) currently represents one of the most pressing health concerns of our time. Worldwide, CHD kills over 7 million people per year and is the cause of one in seven deaths [1]. In the United States, over 16 million are affected by the disease and it is the leading cause of death, killing well over 500,000 yearly [2]. Furthermore, nearly 50% of men and 30% of women over the age of 40 are at risk of developing the disease [2]. The economic burden of the disease in the United States is staggering, with estimated costs of nearly $150 billion in 2006 [2]. As such, the need for efficacious and economical treatment methods for CHD is clearly evident.

CHD is characterized by arterial narrowing (stenosis) due to the accumulation of plaque beneath the endothelium, the layer of cells that make up the innermost lining of the blood vessel. This accumulation, known as atherosclerosis, can cause restriction of blood flow, thus depriving the heart muscle of oxygen. In severe cases, stent implantation has become the preferred method for restoring normal blood flow. Stents are metallic, lattice-like scaffolds that are implanted into stenosed arteries to maintain vascular patency. In their first embodiment, bare metal stents (BMS) simply served as mechanical supports to prevent vascular recoil after balloon angioplasty. However, they have since evolved to incorporate biological functionality as well, in order to mitigate neointimal hyperplasia, the vascular response to implantation-induced injury. This response is characterized by excessive tissue proliferation within the lumen, which can cause re-narrowing over time (restenosis).

Of the numerous solutions developed to address restenosis, the most successful to date has been the drug-eluting stent (DES), which suppresses hyperplastic response via slow release of antiproliferative or immunosuppressive drugs from a polymer coating applied to the stent. There is growing evidence, however, that potential for thrombogenic stimulus (clotting) is higher in DES than BMS, particularly after drug-elution has ceased. While incidence of this so-called late stage thrombosis in DES is generally low (< 5%), there is cause for concern nonetheless because fatality in such cases can be as high as 45% [3]. Although the mechanism has yet to be fully understood, the cause is likely multifactorial, with delayed intimal healing caused by inhibition of endothelialization potentially playing a significant role [4]. This therefore provides the impetus to develop new therapeutic strategies that facilitate rather than delay healing.

A number of studies have begun to show the promise of pro-healing strategies. All presume that rapid restoration of the endothelium over the stent will enable its isolation from circulating blood, and therefore reduce potential for thrombosis. To date, most have focused on use of bioactive agents, such as growth factors, peptides, and antibodies, which are delivered locally via coating-based elution, surface-immobilization, or porous balloon catheter. However, the efficacy of such approaches is often critically dependent upon spatial and/or temporal control of delivery. Moreover, consideration must also be given to the potentially detrimental consequences of unintended systemic exposure [5]. Thus, there is still distinct need for development of more robust and reliable solutions. In this paper we demonstrate preliminary evidence supporting the feasibility of a new therapeutic strategy that seeks to promote healing via rational design of the surface topography at the nanoscale.

INFLUENCE OF NANOSCALE TOPOGRAPHY IN CELL-SUBSTRATE INTERACTIONS

Numerous studies have demonstrated the often beneficial effects of nanoscale topography on cellular function in a variety of implant applications. With regard to cardiovascular applications and endothelial cells in particular, studies have shown distinct improvements in cellular behavior on nanostructured surfaces, including enhanced adhesion, spreading, and migration [6-12]. It has also been demonstrated clinically that, contrary to prevailing belief, nanoscale surface roughness may actually decrease hyperplastic response slightly [13].

In each of the above mentioned studies, surface topography was essentially random in nature, with only limited control over the distribution of feature sizes, their spatial arrangement, or their morphology (i.e. shape). However, cellular response is known to be strongly affected by small variations in each of these parameters, thus suggesting the need for greater control to elicit more uniform and reproducible response. It has also been shown that specifically-shaped surface features may elicit desirable cellular responses. For example, arrays of sub-micrometer scale linear grooves have been shown to enhance endothelial alignment

and elongation significantly compared to micropatterned surfaces [14], thus providing the potential for improved mimicry of the native endothelium. Moreover, micrometer scale linear groove arrays have been shown to produce significant acceleration of endothelialization, due to alignment-induced reduction of contact inhibition between cells [15]. Since many of these responses are observed to scale favorable with decreasing feature size, this suggests that precisely-controlled and rationally-designed topographical variation of the stent surface at the nanoscale may provide considerable promise for enhancing healing and endothelial function in vascular stenting.

EXPERIMENTAL METHODS

Patterned substrate fabrication

Polished titanium wafer substrates (99.6% Ti, 100 mm diameter, 500 µm thickness, R_a ~ 10 nm RMS), were cleaned in ultrasonic baths of acetone and isopropanol, followed by deionized (DI) water rinsing. The wafers were then dried with N_2 and dehydration baked. The wafers were primed with hexamethyldisilazane, coated with photoresist, and pre-exposure baked. The coated wafers were then lithographically exposed using a wafer stepper. After exposure, the wafers were post-baked, developed, rinsed in DI, and dried with N_2.

Each die pattern was composed of an assemblage of 5 x 5 mm sub-patterns, each made up of a periodic array of parallel lines and space of equal width. The sub-patterns were arranged such that they were orthogonally aligned to their nearest neighbors. A different line width was used for each sub-pattern (ranging from 0.75 µm to 100 µm), thus providing the opportunity for simultaneous evaluation of a broad pattern parameter space within the same sample and, therefore, within the same cell culture conditions. The range of chosen feature sizes was bounded at the lower end by the resolution limit of the wafer stepper, and at the upper end by the recognition that the widths of stent struts are typically on the order of 100-150 µm.

The photoresist patterns were transferred into the underlying titanium substrates using inductively coupled plasma reactive ion etching (conditions: 100 sccm Cl_2, 5 sccm Ar, 400 W source power, 100 W substrate power, 2 Pa chamber pressure) [16]. Afterwards, the residual photoresist was removed in ultrasonic solvent baths, followed by DI rinsing and N_2 drying. Resulting groove depths of ~2 µm were achieved with etch times of 2 – 3 min. Due to the limited scope of this preliminary study, groove depth was not varied. However, it is anticipated that groove depth may strongly influence cellular response.

Substrate characterization

Scanning electron microscopy was used to characterize the patterned Ti substrates. Prior to imaging, the substrates were sputter-coated with a thin layer of gold-palladium (these samples were not used for subsequent cell culture experiments). Surface topography was further characterized by atomic force microscopy. Commercially available AFM tips (radius of tip curvature less than 10 nm) were used in tapping mode. Tips with full tip cone angle less than 10° in the last 200nm of the tip apex were used (25 µm full tip height, 30º full cone angle after initial 200 nm, 40 N/m force constant). Scan rate was fixed at 0.5Hz.

Cell culture

Before cell culture experiments, all substrates were rinsed in acetone for 5 min, ethanol for 5 min, and finally in DI water for another 5 min. They were then sterilized by autoclaving (115-120 ºC) for 30 min. Competitive endothelial and vascular smooth muscle cell proliferation on patterned titanium substrates was evaluated in the following manner: endothelial and vascular smooth muscle cells previously incubated with fluorescent stains were suspended in cell culture media (DMEM containing 10% FBS), seeded randomly (2,500 cells/cm^2 surface area) onto the substrate surface, and cultured under standard static cell culture conditions for periods ranging from 4 hours to 5 days. At the conclusion of the experiments, non-adherent cells were removed by rinsing with phosphate-buffered saline. In all experiments conventional wrought Ti surfaces (R_a=4.1 nm) served as controls representative of conventional stent surfaces. Endothelial monoculture assays (i.e. endothelial cells alone) were also performed using the above-listed procedures.

Cellular adhesion and proliferation characterization

Adherent cells were fixed and counted using florescent microscopy. Cell preparations were examined using a fluorescence microscope with cell density (cells per unit surface area) determined by averaging the number of cells in five random fields. Proliferating cells were also visualized using computer-based image analysis. Special attention was given to analysis of cell morphology on the patterned substrates. All cell experiments were run in triplicate and repeated a minimum of three times per substrate type. Numerical data was analyzed using Analysis of Variance (ANOVA) with proliferation as the observed response; values of $p < 0.05$ were considered significant difference between substrate types.

Endothelial cell morphology after culture was also quantified using SEM. Endothelial cells were fixed with formalin for 5 min, then dehydrated by soaking serially in 10%, 30%, 50%, 70%, and 90% ethanol for 30 min, followed by soaking in 100% ethanol for 15 min three times. The substrates were then critical point dried before visualization.

RESULTS

Figures 1 & 2 illustrate the highly precise and anisotropic nature of the grooved surface topography of the patterned titanium substrates. The results of 3 day competitive endothelial and vascular smooth muscle cell proliferation assays on the patterned titanium substrates are shown in Figure 3. The data clearly show that decreasing feature sizes promote strong enhancement of endothelial proliferation and concurrent inhibition of smooth muscle cells; both of which are critical to improving vascular stent efficacy and safety. This stands in stark contrast to the behavior observed on representative conventional stent surfaces (i.e. wrought Ti control samples), where endothelial proliferation is strongly inhibited relative to smooth muscle cells.

Endothelial monoculture assays produced similar trends of enhanced response with decreasing size scales. Endothelial spreading behavior under 3 day monoculture conditions is shown in Figure 4. Increasing cell density, better spreading, and greater alignment was observed with decreasing feature size. Figure 4(d) provides further evidence of strong contact guidance, as indicated by the sharp transition of cellular alignment from one orthogonally aligned sub-pattern to the next. A scanning electron micrograph of endothelial spreading after 3 day monoculture on a 0.75 µm patterned substrate is shown in Figure 5 and further demonstrates the strong alignment induced by the sub-micrometer patterned surfaces. Additional monoculture experiments (data not shown) also demonstrated that time to endothelial confluence decreased with decreasing feature size. Specifically, confluence was achieved within 3 days on 0.75 µm features, whereas confluence was not achieved even after 5 days on wrought Ti control surfaces. Collectively, these data suggest potential for strong affect of nanopatterning on endothelialization *in vivo*.

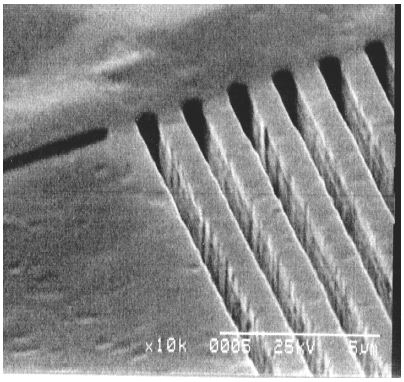

Figure 1: Scanning electron micrograph (inclined view) of patterned titanium substrate with 0.75 µm wide grooves and space. Scale bar = 5 µm.

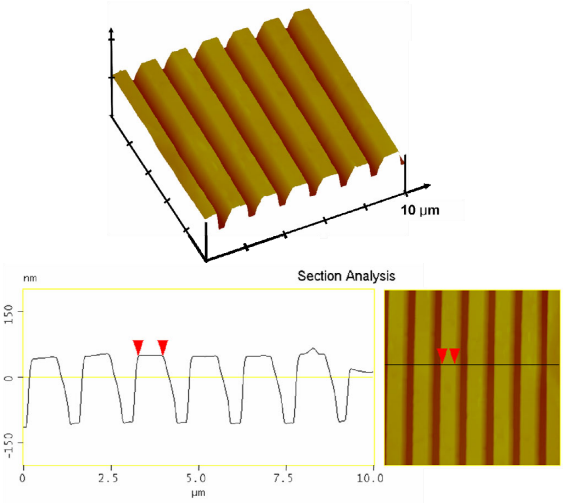

Figure 2: Atomic force micrographs of patterned titanium substrate with 0.75 µm wide grooves and space.

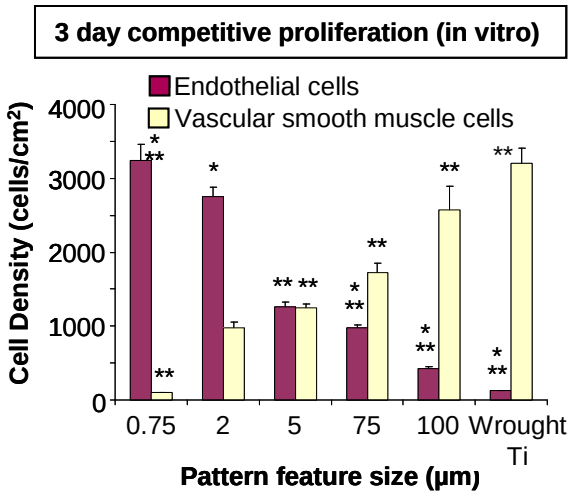

*Figure 3: 3 day competitive cell proliferation on patterned titanium substrates as a function of feature size. Feature sizes correspond to groove width and spacing. Data = mean +/- SEM; N =3; * p < 0.01 (compared to vascular smooth muscle cells on same feature dimension); ** p < 0.01 (compared to 2 µm features).*

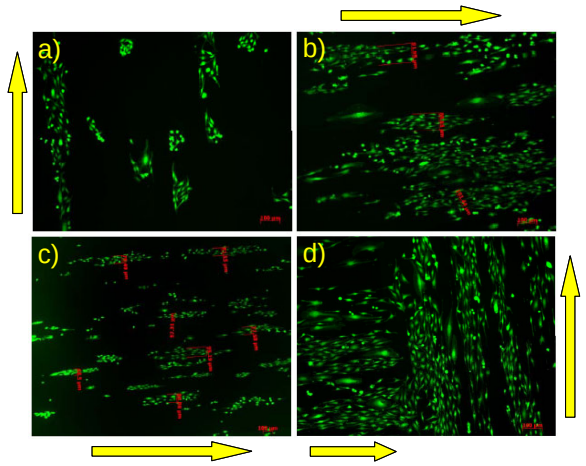

Figure 4: Endothelial spreading (live, stained) for 3 day monocultures on patterned titanium substrates with: a) 100 µm; b) 25 µm; c) 2 µm; and d) 0.75 µm grooves & space. Arrows indicate approximate direction of pattern alignment. Scale bar at bottom right of each image = 100 µm.

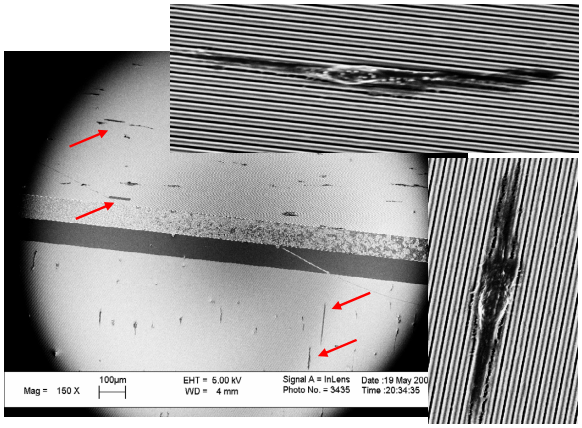

Figure 5: Scanning electron micrographs of 3 day monocultured endothelial cells adhered to patterned titanium substrate with 0.75 µm grooves and space. Background: Low magnification view of two adjacent patterned regions; the upper is horizontally aligned, while the lower is vertically aligned. Arrows mark examples of highly aligned cells. Foreground: High magnification views demonstrating strong endothelial alignment along ridge tops of two patterns.

DISCUSSION

This preliminary study represents the first demonstration of enhanced competitive endothelial cell response relative to vascular smooth muscle cells on patterned titanium substrates. Clear evidence is presented of improved endothelial proliferation, higher cell density, and greater alignment with decreasing pattern feature size, particularly as feature sizes are pushed into the sub-micrometer realm. This suggests that subsequent cellular function may be improved with decreasing feature size. Perhaps more importantly, however, it also provides compelling first evidence of the potential for mitigation of restenosis and thrombosis solely through rational design of the surface topography of the stent material itself. As such, the inherent simplicity, robustness, and reliability of this approach is immediately apparent, as is its potential for improving the efficacy and safety of vascular stents.

The exact mechanisms by which such surface features affect cellular response are still unknown, however prevailing theories suggest that they may either: a) modulate the interfacial forces that guide cellular structure and intracellular signaling [17]; or b) influence the nature of protein adsorption on the surface, thus affecting the expression of integrin receptors that regulate subsequent cellular adhesion and function [18]. With regard to vascular cells in particular, recent evidence has been reported supporting the latter hypothesis. It was noted that adsorption and unfolding of fibronectin, a key protein involved in endothelial cell adhesion, were both enhanced on randomly nanostructured surfaces of biodegradable polymers [6,19]. The same results have since been observed for vitronectin. It would therefore be presumed that endothelial response would be enhanced by not only the increased number of binding sites produced by higher concentrations of adsorbed protein, but also by their more favorable conformation, since unfolding may promote expression of key cell-adhesive integrin receptors, such as RGD. However, vascular smooth muscle cells also adhere to RGD contained in fibronectin and vitronectin, yet their competitive adhesion is observed to decrease with decreasing feature size. This suggests that competitive pressure from endothelial cells may play a strong role in inhibiting smooth muscle cell proliferation on patterned titanium surfaces.

Although enhanced competitive endothelial response was observed with decreasing feature size in the preliminary study, the smallest features probed were still only within the sub-micrometer range, i.e. 0.75 μm and above. It is well known, however, that many important cellular and extracellular matrix components are of far smaller dimensions, with most within the range of tens to a few hundreds of nanometers [20]. Filopodia, are one particularly important example within the context of cell-substrate interactions. These tendril-like cellular projections are used probe the substrate in advance of establishment of focal adhesions. They have been shown to preferentially attach to nanoscale surface features [9] and it has been suggested they may be responsible for cellular alignment along patterned features (i.e. contact guidance) [14]. They have also been shown to increase in number in response to nanoscale surface features [21]. Consequently, this suggests that further reduction of feature sizes in the patterned titanium substrates may increase filopodia-based interactions which may then yield even greater endothelial response. As such, the implications for vascular stents are enormous. Improved competitive endothelial proliferation, higher cell density, and improved alignment might enable rapid and robust endothelialization of the stent, thus reducing potential for thrombosis and restenosis.

CONCLUSION

The current study represents the first demonstration of nanopatterning-enhanced endothelialization in materials of immediate relevance to vascular stents. Furthermore, the current study presents the first demonstration of strongly enhanced competitive endothelial proliferation over vascular smooth muscle cells on nanopatterned surfaces. Finally, the current study represents the first demonstration of such behavior in patterned metallic surfaces created using fabrication techniques that are intrinsically scalable to low-cost/high-volume manufacturing of vascular stents. This is a particularly key distinction, since it demonstrates a clear path towards clinical translation. Consequently, this study provides compelling evidence of a potentially robust and reliable new means of improving the safety and efficacy of vascular stents.

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