Enhancement of In Vitro Capillary Tube Formation by Substrate Nanotopography

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ABSTRACT

Tissue engineering scaffolds often aim to control cell behavior using a variety of signaling modalities including chemistry, mechanical properties, and local microenvironmental factors such as oxygen concentration. However, few efforts have explored the use of nanotopography as a means to regulate cell function. Controlling the morphology and function of cells using substrate nanotopography is a phenomenon that can be utilized in a variety of fields including tissue engineering and regenerative medicine. In this work, we explored the possibility of using nanofabricated surfaces to control the function of endothelial cells to ultimately enhance in vitro vasculargenesis. We chose to culture endothelial progenitor cells (EPCs) on poly(dimethylsiloxane) substrates with ridge-groove geometries of approximately 600 nm in width. EPCs cultured on nanotopographic were found to exhibit enhanced morphological alignment and elongation, reduced proliferation, and enhanced migration. Protein-level expression of endothelial cell markers was not significantly affected by topography, as determined by fluorescent microscopy. However, an in vitro capillary tube formation assay induced the formation of larger, more organized vascular structures in EPCs cultured on nanotopographic versus flat substrates. These results suggest substrate nanotopography could function as a tool for controlling EPC function and achieving enhanced vasculargenesis. Furthermore, these nanotopographic substrates could serve as a template for engineering more complex vascularized tissues and organs.

Keywords: nanotopography, tissue engineering, blood vessel

1 INTRODUCTION

Vascular engineering remains a key thrust in advancing the field of tissue engineering of highly vascularized, complex, metabolic organs. A wide variety of strategies have been employed to control the formation of organized vascular structures in vitro and in vivo. Some of these methods include, but not limited to, controlled growth factor delivery [1], filamentous scaffold geometry [2], protein micropatterning [3], and enhanced scaffold biomaterials [4]. Many of these approaches are motivated by biomimicry of the in vivo microenvironment. ECM proteins, both in vitro and in vivo, provide mammalian cells with biophysical cues including specific surface chemistry and rich three-dimensional surface topography [5] with features on the nanometer length scale [6]. ECM substrates provide chemical and physical external cues that dictate a variety of cell responses. Therefore, it is not only the milieu of soluble, diffusible factors, but also the adhesive, mechanical interactions with scaffolding materials, both natural and synthetic, that control select cell functions including cell attachment, migration, proliferation, differentiation, and regulation of genes [7-9]. We hypothesized that physical features on nanofabricated substrates could promote the organization of endothelial cell lineages into well-defined vascular structures in vitro by inducing the contact guidance phenomenon, which is known to affect the morphology of endothelial cells [10-12]. We found that endothelial progenitor cells (EPCs) responded to ridge-groove grating of 1200 nm in period and 600 nm in depth through alignment, elongation, reduced proliferation and enhanced migration. Although endothelial-specific markers were not significantly altered, EPCs cultured on substrate nanotopography formed supercellular band structures after 6 d. Furthermore, an in vitro matrigel assay led to enhanced capillary tube formation and organization.

2 RESULTS

Feature geometry and dimensions of nanotopographic PDMS substrates were verified by SEM. The 600 nm width for ridge and groove features was chosen to promote optimal contact guidance effects in EPCs by minimizing feature masking from the collagen coating and maximizing cell alignment through sub-micron features. The EPCs seeded as individual cells on nanotopographic substrates and responded to linear nanotopography by alteration in morphology as observed by increased alignment and elongation (Figure 1). These alterations were quantified by reduced average angle of alignment and circularity at 2 and 4 d. SEM imaging confirms that the EPCs were aligned an elongated in direction of substrate features. Furthermore,

the morphological alterations were maintained throughout long-term culture on nanotopographic substrates for up to 6 d. EPCs cultured on nanotopographic substrates also exhibited reduced proliferation as measured by BrdU assay and reduced cell growth kinetics as determined by cell density compared to EPCs cultured on flat substrates. The observed doubling time was 16.2 ± 0.8 to 20.9 ± 1.9 h for cells grown

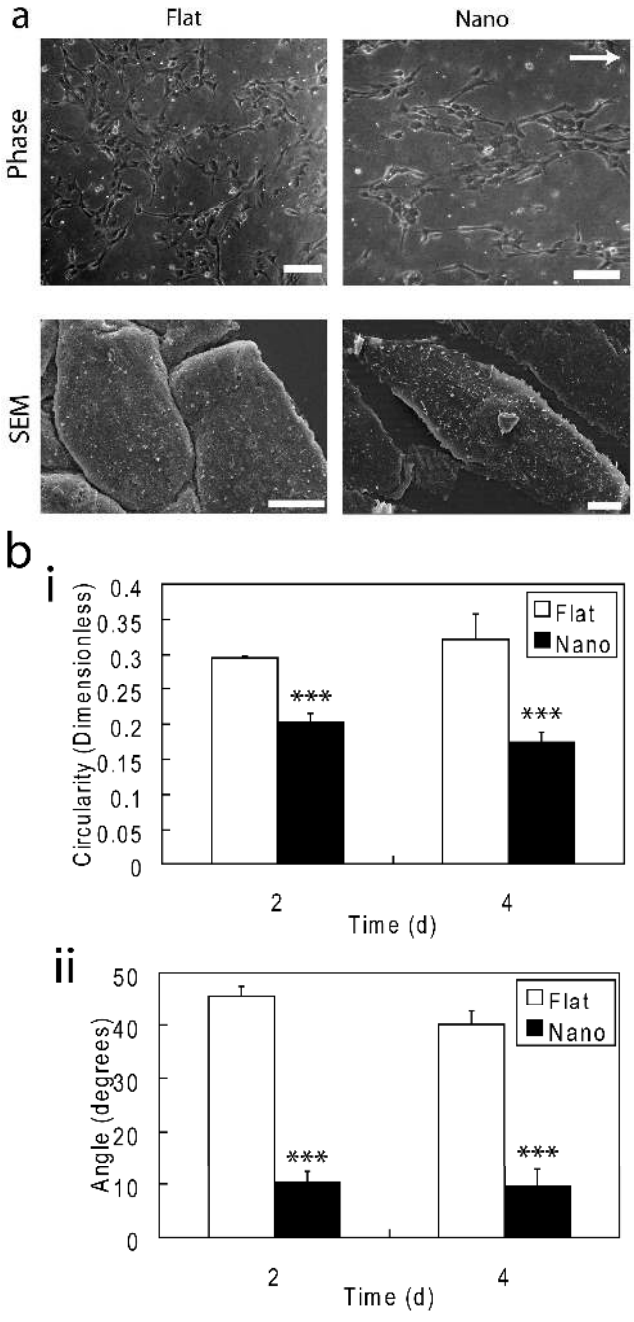

Figure 1: (a) Optical and SEM micrographs of EPCs cultured on synthetic nanotopographic substrates after 24 h. (b) Quantitative morphology demonstrate that EPCs are elongated and aligned with nanotopographic structures as determined by reduced circularity and increased alignment.

on flat and nanotopographic substrates, respectively. A third component of the contact guidance response that was observed in EPCs was enhanced migration. EPCs on nanotopographic substrates exhibited a higher overall migration velocity as well as enhanced directed migration, as measured by effective migration distance. The average velocity of EPCs on nanotopographic and flat substrates was 0.80 ± 0.45 and 0.54 ± 0.27 μm-min^{-1} (*** $p < 0.001$), respectively, while the effective displacement due to migration was 23.6 ± 12.1 and 15.6 ± 10.1 μm (*** $p < 0.001$), respectively.

Linear nanotopographic substrates organized populations of EPCs into band structures at 6 d consisting of hundreds of cells and extending for millimeters in length. The supercellular bands contained a well-defined edge that paralleled the feature grating. This edge was identified by EPCs with highly elongated, constrained morphology. These band-like structures were distinct from each other and did not merge to form confluent monolayers of cells. These gross morphological changes were observed for up to 6 d. The morphology of EPCs cultured on nanotopography lies in stark contrast to EPCs cultured on flat substrates, which did not form supercellular structures and instead produced confluent monolayers of EPCs after 6 d. Despite the marked alterations in morphology, proliferation, and migration states, the level of protein-level expression of selected markers was observed to be similar across substrates, as assessed by immunohistochemistry at 6 d. These markers included CD31 (PECAM-1), vascular endothelial cadherin (VEcad), and α-smooth muscle actin (α-SMA, negative staining) (Figure 2). Additional markers that were similarly expressed in cells cultured on both substrates include von Willebrand factor, VEGF-2 receptor (KDR), and lectin receptor. The effects of nanotopography on morphology and endothelial-cell specific marker expression were observed to be independent. The addition of matrigel induced capillary tube formation in EPCs cultured on both substrates in less than 4 h as assessed by light microscopy. EPCs cultured on flat substrates formed short, randomly oriented capillaries while EPCs cultured on nanotopography formed well-defined capillary tubes with increased length (Figure 3). Furthermore, capillary tubes on nanotopographic substrates exhibited enhanced alignment and organization. The fraction of EPCs recruited into capillary tubes was also increased dramatically in cultures with substrate nanotopography. This observation contrasted with the observed tube formation in EPCs culture on flat substrates, which was characterized by a high density of tubes with short lengths oriented in random directions. Furthermore, only a small fraction of cells cultured on flat substrates participated in capillary tube formation. Although the three-dimensional morphology of cells was not explicitly studied in this work, others have shown that the culture of endothelial cells in the presence of matrigel induces the formation of three-dimensional capillary tubes with lumens [13, 14].

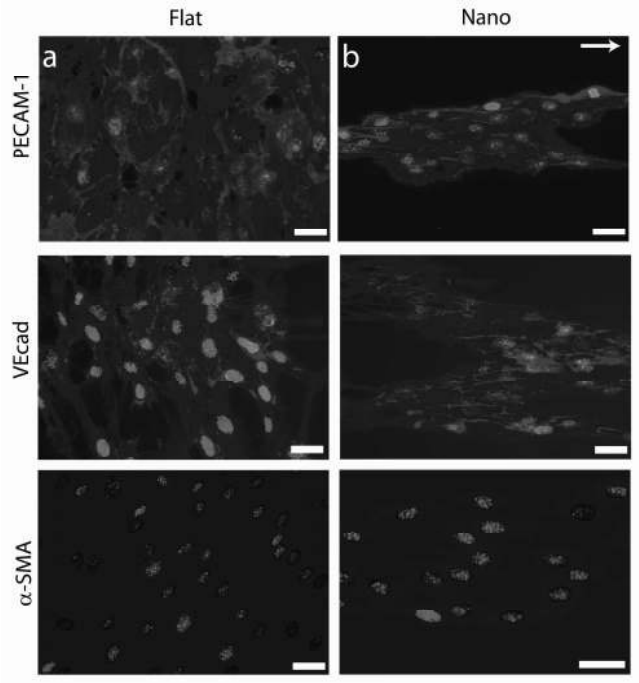

Figure 2: Nanotopographic substrates induced formation of multicellular band structures without altering protein-level expression of endothelial cell markers.

3 DISCUSSION

The observed cell response of EPCs to linear substrate nanotopography in this study is in concert with previous work on a variety of substrate materials and cell types. Alignment of cells to linear micron and sub-micron scale features is a well-characterized response that occurs in many different cells types including endothelial cells [10]. Reduced proliferation has also been observed in a variety of cell types including smooth muscle cells and human embryonic stem cells [15, 16]. The increased migration velocity of EPCs on substrate nanotopography is also in agreement with previous work of various mammalian cell types including corneal epithelial cells [17]. This collective response is a principle element in the in vitro contact guidance with substratum cues. One result of note regarding this aspect of EPC responses to nanotopography is that the morphological changes were maintained for long-term culture. Oftentimes the elongated morphology becomes affected as cell growth leads to impingement of protrusions. The maintenance of this morphological aspect can be attributed to the formation of aligned bands of cells which enabled preservation of the elongated morphology.

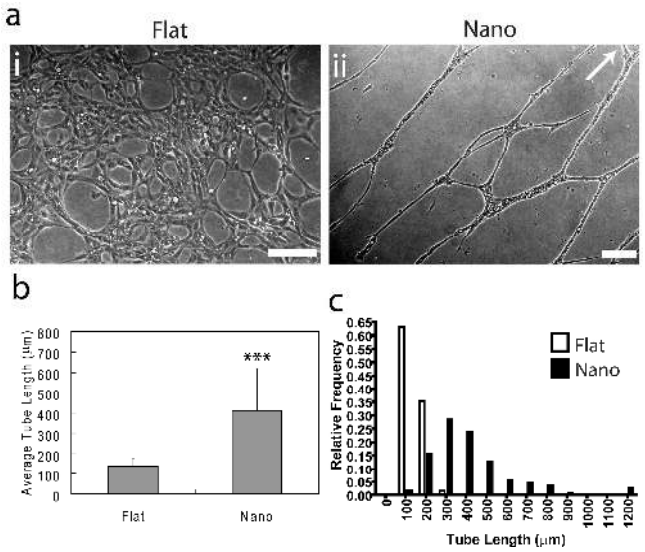

Figure 3: Nanotopographic substrates induced formation of well defined, organized capillary tubes that are greater in length. Endothelial cells cultured on nanotopographic substrates are recruited into capillary tubes more efficiently as well.

Cells have been shown to respond to substrate nanotopography at the protein level as well [18]. While it is likely that nanotopography impacts the genetic profile of EPCs, the expression profile of selected markers was similar in EPCs cultured on both substrates, which implies that surface nanotopography has no significant impact on the protein level of endothelial-specific markers. Despite this observation, the impact of nanotopography on the overall organization of EPCs and the enhanced in vitro capillary tube formation was maintained. The nanotopographic features were hypothesized to play a governing role in this observation via the enhanced formation of band structures primarily through two means. First, linear nanotopographic features align and elongate individual EPCs which ultimately form clusters of EPCs. This clustering is hypothesized to promote cell-cell interactions, which ultimately lead to the formation of the observed band structures. Second, the reduction in proliferation prevents the growth of EPCs into confluent monolayers of cells on nanotopographic substrates. The unoccupied surface area is hypothesized to enable the rapid formation of capillary tubes in vitro. Conversely, the formation of confluent layers, as observed in cells cultured on flat substrates, possibly retards the formation of capillary tubes because of the spatial constraint of EPCs interfering with each other.

The observations in this study further suggest the continued application and integration of nanotopographic features in tissue engineering systems. For example, biodegradable polymers amenable to soft-lithography could be used in future studies [10]. Furthermore, other vascular progenitor cells and co-culture systems could also be employed to explore the use of systems with physical

surface cues for therapeutic applications [19]. This system could also be directly used to study the mechanisms of vascular genesis by investigating the cellular pathways involved in the observed enhanced capillary tube formation. The introduction of co-cultures could potentially serve as a platform to elucidate the underlying homotypic and heterotypic cellular processes that are biologically relevant to blood vessel formation in vitro and in vivo [20].

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