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Azopolymer photopatterning for directional control of angiogenesis

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ABSTRACT

Understanding cellular behavior in response to microenvironmental stimuli is central to tissue engineering. An increasing number of reports emphasize the high sensitivity of cells to the physical characteristics of the surrounding milieu and in particular, topographical cues. In this work, we investigated the influence of dynamic topographic signal presentation on sprout formation and the possibility to obtain a space–time control over sprouting directionality without growth factors, in order to investigate the contribution of just topography in the angiogenic process. To test our hypothesis, we employed a 3D angiogenesis assay based on the use of spheroids derived from human umbilical vein endothelial cells (HUVECs). We then modulated the *in situ* presentation of topographical cues during early-stage angiogenesis through real-time photopatterning of an azobenzene-containing polymer, poly (Disperse Red 1 methacrylate) (pDR1m). Pattern inscription on the polymer surface was made using the focused laser of a confocal microscope. We demonstrate that during early-stage angiogenesis, sprouts followed the pattern direction, while spheroid cores acquired a polarized shape. These findings confirmed that sprout directionality was influenced by the photo-inscribed pattern, probably through contact guidance of leader cells, thus validating the proposed platform as a valuable tool for understanding complex processes involved in cell-topography interactions in multicellular systems.

Statement of Significance

The complex relationship between endothelial cells and the surrounding environment that leads to formation of a newly formed vascular network during tissue repair is currently unknown. We have developed an innovative *in vitro* platform to study these mechanisms in a space and time controlled fashion simulating what happens during regeneration. In particular, we combine a "smart" surface, namely a polymer film, with a three-dimensional living cell aggregate. The polymer is activated by light through which we can design a path to guide cells toward the formation of a new vessel. Our work lies at the intersection of stimuli-responsive biointerfaces and cell biology and may be particularly inspiring for those interested in designing biomaterial surface related to angiogenesis.

1. Introduction

Tissues house an extensive blood vessel network that is able to regenerate after injury by endothelial cell (EC) sprouting from the existing vasculature. This process, called angiogenesis, requires the temporal and spatial coordination of different cellular events elicited by microenvironmental conditions such as hypoxia and inflammation [1,2]. Recent developments highlight that ECs are particularly influenced by features of the extracellular matrix (ECM) and of the basal membrane, to which ECs are anchored [3,4]. All these biochemical and physical signals initiate a cascade of events promoting a variety of cellular processes such as cell phenotype selection, i.e. tip and stalk, migration, proliferation and tubulogenesis. Tip cells sense the environmental changes and are the leading cells that guide the following stalk cells through the process of sprout formation [5]. In addition, revascularization occurring after an injury is often too slow to guarantee

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proper O_2 and nutrient supply to the regenerating tissue [1]. For this reason it is essential to create engineered biomaterials capable of reproducing *in vitro* the dynamic nature of the ECM [3] so as to unravel the mechanisms underlying the cell-material interplay for a controlled revascularization in pathological conditions. Several strategies in tissue engineering and regenerative medicine involve the formation of a vascular network within a biomaterial, which is then expected to integrate with the host tissue [6]. However, the production of biomaterials capable of encouraging the onset of a functional vascular network avoiding the formation of irregular neo-vessels remains an open challenge [7]. In fact, often the newly formed vessels are tortuous and fenestrated, with an increased probability of implant failure due to turbulent blood flow and to an insufficient supply nutrients to the regenerating tissue [7,8]. In this scenario, new approaches in biomaterial design and surface bioactivation may be helpful to address these issues.

Over the past few years several models based on cells combined with biomaterials have allowed for the study of vessel branching mechanisms *in vitro* [9]. Many reports have characterized the influence of soluble factors on the directional movements of ECs as well as the role of biomolecules involved in angiogenesis, yet very little is known about the direct effect of the topographical features of the ECM on the formation of a functional vessel network. Indeed, it is well-known that topographical cues play an important role in influencing cell behavior and fate. For example, it has been shown that anisotropic alignment of ECM fibers due either to cell traction forces, or by means of external stretching, provides the so-called contact guidance during the formation of endothelial sprouts [10]. However, the current approaches are primarily based on the use of static materials, which are unable to modify their features during cell culture. Thus, they fail in addressing the issue of the spatio-temporal presentation of topographical cues, which is crucial in recapitulating *in vitro* the dynamic nature of ECM [9,11,12]. In fact, the natural environment where the cell exists is dynamic in that the chemical and physical properties of the ECM are continuously changing around the cells. In this respect, smart systems including biomaterials activated through external stimuli at any specific time during cell culturing may provide insights into the mechanisms underlying the dynamic cell-material interaction [10]. In particular, light is an advantageous external trigger allowing fine spatio-temporal control in a contactless fashion, reducing the risks of sample contamination. Therefore, we based our hypothesis on the fact that "switching on" the topographical signal at a specific time may impact sprout formation, in part through contact guidance of leader cells. In fact, the use of azobenzene-based polymers has recently been recognized as a successful approach to change topography in real-time in the presence of living cells exploiting light [13,14]. In this context, Poly(Disperse Red 1 methacrylate), here named as pDR1m, is a well-known azopolymer, which is widely used in photonic applications for its unique ability to modulate the material surface profile by a light-induced mass migration. In addition, it has recently been used for biological applications [13–18]. Based on this background we used a pDR1m photoresponsive thin film to test our hypothesis on the role of topographical modification during angiogenesis. To this purpose we coupled the use of pDR1m to a well-established endothelial cell spheroid-based angiogenesis model [19–22]. In our system collagen does not embed the spheroids but just covers them in order to support tubule formation in a semi-3D setting. Thus, through material photopatterning generated by a confocal microscope, we were able to deliver *in situ* a topographical cue during early-stage angiogenesis while observing sprout rearrangement in real-time.

2. Materials and methods

2.1. Substrate preparation

For the fabrication of the dynamic substrate, poly(Disperse Red 1 methacrylate), pDR1m (Sigma-Aldrich), was dissolved in chloroform (5% w/v) and then spin coated onto a glass coverslip (15 mm diameter). While, to make the static topography (used as patterned control sample), NOA63 (Norland Optical Adhesive, photocurable polyurethane purchased by Norland Products) was imprinted by replica molding using a patterned polycarbonate master and polymerized under UV light exposure for 20 min (Fig. S1). The resulting linear pattern (2.5 μm pitch and about 140 nm high) covered the entire sample surface. A custom-made single-well plate was built, in which patterned NOA63 or a pDR1m thin film was positioned at the bottom of each well and a polystyrene hollow cylinder (10 mm internal diameter) was then glued on each well using polymerized NOA63 after UV light exposure (Fig. S2). Both bare glass and spin coated azopolymer films not treated with laser light are referred as "flat" control samples. Additionally, in the case of patterned NOA63 samples, a flat NOA surface was used as control sample as well. All samples were characterized by atomic force microscopy (AFM) using a JPK NanoWizard II (JPK Instruments), mounted on the stage of an Axio Observer Z1 microscope (Zeiss). Commercial silicon nitride tips (MLCT, Bruker), with a resonance frequency of 50 kHz and a spring constant of 0.10 N/m, were used to scan in contact mode, in air, all over the sample area of interest.

2.2. Cell culture and generation of endothelial spheroids

Human umbilical vein endothelial cells (HUVECs) (Lonza) were grown in Medium 200 supplemented with LSGS kit (Life-Technologies) containing fetal bovine serum (FBS) 20% v/v, hydrocortisone 1 μg/ml, human epidermal growth factor 10 ng/ml, basic fibroblast growth factor 3 ng/ml and heparin 10 μg/ml at 37 °C in 5% CO_2 and 100% relative humidity. Cells were seeded into tissue culture flasks and cultured until they reached confluence. To generate endothelial spheroids, they were used at early passages (II-IV).

After 3–4 days of culture, confluent HUVEC monolayers were trypsinized and 800 cells per spheroid were suspended in Medium 200 containing 3 mg/ml carboxymethylcellulose (Sigma) and supplemented with LSGS kit with 5% of FBS, seeded into ultra-low-attachment u-bottom 96 well plates (Costar) and cultured at 37 °C in 5% CO_2 and 100% relative humidity to allow spheroid formation [23].

2.3. Imaging sprouting angiogenesis assay on patterned surfaces

After 24 h of culture, spheroids were harvested and centrifuged at 900 rpm for 15 min. Then, 1–2 spheroids were seeded on a polymer coated glass slide (custom-made single-well system). Afterwards, they were covered with 1.2 mg/ml of bovine skin collagen in a volume of 80 μl per well. After collagen gelation at 37 °C we left the samples in the incubator for 30 min. Through this procedure the collagen self-assembled into fibrillar structures. Afterwards, M200 culture medium, supplemented with LSGS kit and HEPES 25 μl/ml (Sigma) was added. Polymer surface was then photopatterned for 45 s by using a Leica SP5 confocal microscope equipped with a 25x water immersion objective (focal depth of 1.3 μm) and an Argon laser set at 514 nm wavelength and a temperature-controlled chamber. Rectangular regions of interest (ROIs) were drawn (200 μm x 2 μm), giving a final pitch of 3 μm, directly underneath each spheroid. Spheroids belonging to the control group (see section 2.1) were seeded in the same conditions on

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