RETENTION OF ENDOTHELIAL CELLS UNDER HIGH SHEAR STRESS ON MICROPATTERNED POLYURETHANE SURFACES

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INTRODUCTION

Synthetic (polymeric) vascular grafts have completely revolutionized cardiovascular surgery. However, a common problem with synthetic vascular grafts is the formation of thrombus on the inner wall of the graft. While this event does not affect the patency and performance of large diameter grafts (e.g. aortic grafts) due to their large cross-sectional area; small caliber grafts (<4 mm id.) can get occluded and this represents the primary mode of failure [1]. Studies by Herring et al., in the 1970s, established that endothelial cells (EC) are critical in the prevention of thrombus formation. ECs possess a negatively charged surface that is thought to play a role in the repulsion of platelets [2]. Furthermore, ECs secrete nitric oxide (NO), which has an anti-thrombogenic effect. In view of the beneficial effects of ECs, an obvious solution is the introduction of a viable endothelium. Although several methodologies have been developed to establish a viable endothelium on synthetic graft surfaces (Teflon®, Dacron®, polyurethane) including coating with ECM molecules, chemical modification of graft surface, introduction of surface porosity; the retention of the neo-endothelium in high shear stress environment of arterial circulation has proven to be a challenge [3].

To address the problem of de-endothelialization (EC shedding) of pre-seeded synthetic vascular grafts, we have taken a non-chemical approach. We hypothesized that by creating well-defined micro-patterns on a surface, fluid flow can be altered to create discrete regions of low-no shear stress. We further hypothesized that due to reduced stress; EC retention in these discrete regions will be enhanced. To verify this hypothesis we studied the retention of bovine aortic endothelial cells (BAEC) on polyurethane (PU) surfaces that were patterned with an array of alternating micro-channels and compared them to unpatterned surfaces. Our thinking behind using such a pattern was that the creation of alternating plateaus (P) and valleys (V) would allow for direct comparison between adjacent regions of differing stress and serve as a rigorous model system to verify our hypothesis.

MATERIALS AND METHODS

Preparation of pattern template

A negative impression of the desired pattern of alternating closed channels was created on a silicon wafer (4 cm) substrate using standard lithography techniques.

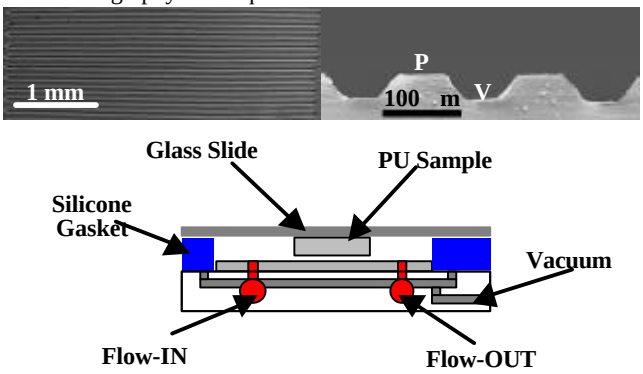

Figure 1. Top Panel: SEM image of patterned PU film surface. Left: Channel arrangement; right: Cross-sectional view of the film, plateau (P) & channel valley (V). Bottom Panel: Schematic of flow chamber.

Preparation of μ-patterned polyurethane (PU) films

The pattern was transferred onto medical grade PU films by solvent casting technique (Figure 1, top panel). In brief, a warm solution (45° C) of PU in THF (75 mg/ml) was deposited on a silicon wafer template in a drop-wise manner until complete surface converge was achieved. The film was air-dried for 12 hours and released from the silicon substrate by soaking in isopropanol. Non-patterned PU films were made by a similar casting procedure on virgin silicon wafers. Under these conditions films ~160 μm in thickness were obtained. The patterned films were cut into 16 mm squares; each square represents four arrays of channels (Figure 1, top left) (each

4mm x 5mm). PU films were sterilized by immersion in 70% ethanol for 30 minutes.

Assembly of PU films for flow experiment and EC seeding

PU films were assembled onto to the center of sterile glass slides (Fisher Scientific, #12-550B) using sterile vacuum grease such that the length of the channel (major axis) was parallel to the direction of flow. The assembly was carried out in a laminar flow cell culture hood to ensure sterility. The film-slide assemblies were placed in 10 cm petri dishes (Falcon, #353003) and the PU surface was coated with a 100 µL drop of fibronectin solution in 1x PBS (100 µg/ml) and air-dried for 1 hour. Once the drop dried, the PU-slide assembly was bathed in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (culture medium) and left in the incubator for 12 hours until EC seeding. Bovine Aortic Endothelial Cells (BAEC) were isolated as described in the literature [4] and cultured in T75 flasks in culture media. P3-P5 ECs at a density of 400K cells/square were seeded 24 hours prior to the flow experiment. Unpatterned PU film and gelatin (0.1 %) coated glass slides were used as static controls. ECs on all substrates exhibited cobblestone morphology typical of a confluent monolayer.

Flow studies

The flow studies were carried out using a closed-loop circuit comprising of a peristaltic pump, a compliance chamber, media reservoir placed in a 37º C bath and a parallel-flow chamber, in series. The parallel-flow chamber used in this study (Figure 1 bottom panel), produced laminar flow [5]. PU films seeded with ECs were exposed to shear stress of ~60 dynes/cm^2 for a duration of 1 hour. Cells were fixed using 10% paraformaldehyde for 10 mins and then mounted with mounting medium containing DAPI. Images were acquired using a Zeiss fluorescent microscope coupled to a CCD camera, with a 5x objective, using AxioPlane software. A total of 16 images were taken from the center portion of the film for each sample. A corresponding bright field image was taken to visualize channel outlines. The cells were counted manually using a transparency of bright field image as a template. The projected area in a given image was determined using the following conversion factor: 513 pixels = 1 mm^2, which was obtained using a 5x image of a calibrated slide. The projected area for the 'P' and 'V' region was 0.59 and 0.58 mm^2, respectively.

RESULTS

Flow studies were carried out using two different substrates: (1) unpatterned PU and (2) patterned PU. The results are shown in Figure 2A. Polymer substrates not subjected to flow 'static condition' were used as positive controls. We observed that upon exposure of ECs on unpatterned surfaces to flow, density of ECs (cells/mm^2) was diminished by 40±6% from 2198±37 to 1265±218 (n=3). Furthermore, de-endothelialization was observed to occur in a 'patchy' manner. The difference in EC density between the static and flow conditions was statistically significant with a p<0.008.

In the case of the patterned surfaces (Figure 2B), EC densities in the 'P' and 'V' region in controls (static conditions) were statistically identical (n=3, p = 0.18) with cell densities of 1651±235 and 1864±252, respectively with an average P/V ratio of 0.89.

After exposure to flow-induced shear stress, the EC densities in the plateau and valleys were statistically different (n=4, p<0.02) with cell densities of 1171±311 and 2056±334, respectively with an average P/V ratio of 0.56. This constitutes a 29±2 % reduction in cell density in plateaus with respect to patterned controls. In some regions on the polymer surface, a total loss of cells from the plateau region was observed with retention in corresponding valleys (Figure 3). In comparison, the EC density in the valleys after flow (2056±334) was statistically similar (n=4, p>0.2) to densities in static controls (1864±252).

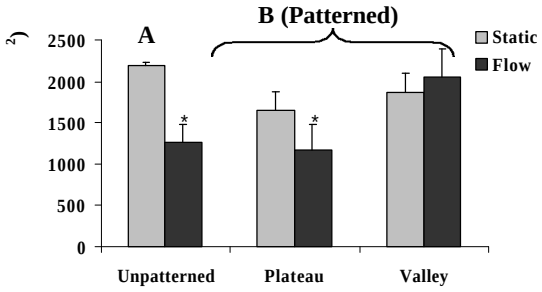

Figure 2. BAEC density on unpatterned (n=3) and patterned (n=4) PU surfaces under static conditions and after flow. Asterisk indicates that flow decreased cell density relative to corresponding static conditions (p<0.008 for unpatterned and p<0.024 for plateau). Error bars represent standard deviation.

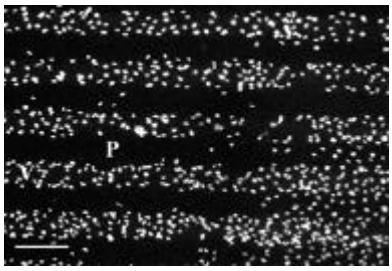

Figure 3. Fluorescent image of BAEC stained with DAPI, a nuclear stain, on patterned PU surface after exposure to flow. Scale bar: 100µm. P = plateau and V = valley.

DISCUSSION

We have demonstrated a novel approach for ensuring retention of ECs under a high shear stress of 60 dynes/cm^2, on synthetic polymer graft surfaces. This was achieved by creating well-defined micro-patterns of valleys (closed channels). Fluid dynamic simulations suggest that under the experimental conditions, stagnation in the channel can occur leading to a low-no stress environment, which is favorable for EC retention. Due to the simplistic nature of this approach, we believe it can be widely applied to include other cell types and surfaces. As shown, with the appropriate choice of valley geometry and spatial distribution high retention of ECs may be achieved.

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