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Sandwiched polymer fibre in fibrin matrices for the dictation of endothelial cells undergoing angiogenesis

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Abstract. We present here a three-dimensional (3D) sandwich system made by poly(ethylene terephthalate) (PET) fibre and fibrin extracellular matrix (ECM) for endothelial cell dictation and angiogenesis guidance. In this three-dimensional system, Human Umbilical Vein Endothelial cells (HUVECs) were firstly cultured for 2 (two) days to cover the PET fibre before sandwiched in two layer fibrin gel containing HUVECs. After 4 (four) days of culture, cel-to-cel connection, tube-like structure and multi-cellular lumen formation were then assessed and validated. Phase contrast and fluorescence imaging using an inverted microscope were used to determine cell-to-cell and cell-ECM interactions. Laser scanning confocal microscopy and histological techniques were used to confirm the development of tube-like structure and multi-cellular lumen formation. This study shows that polymer fibres sandwiched in fibrin gel can be used to dictate endothelial cells undergoing angiogenesis with potential application in cancer and cardiovascular study and tissue engineering vascularisation.

1. Introduction

The ability to dictate endothelial cells undergoing angiogenesis and growing microvasculature containing multi-cellular lumen has been a long-standing challenge in tissue engineering research [1]. Angiogenesis is the formation of new blood microvessel structure from the pre-existing vasculature. It is a key mechanism to promote microvascular network inside engineered tissue construct that mimic the development of blood vessel functions. To date, there are several three-dimensional assays have been developed for angiogenesis study. They are mainly aimed to assess the multiple steps during angiogenesis progression, including: cell-to-cell connection, tube-like structure development, extracellular matrix (ECM) degradation, and multi-cellular lumens formation [1-2].

The formation of microvascular network inside the engineered scaffold will maintain an adequate tissue oxygenation, nutrient transfer and waste removal, thus secure tissue regeneration [2]. One of the main problems faced by tissue engineering researchers now is how find suitable *in vitro* assays and methods to study angiogenesis regulations. Example, some angiogenesis studies failed to report the maturation of tube-like structure and the present of multi-cellular lumen structures [3-4].

This study presents an *in vitro* culture system using polymer monofilament, poly-(ethylene terephthalate) (PET), for the guidance of human umbilical vein endothelial cells (HUVECs) undergoing angiogenesis. Step-by-step developmental process of angiogenesis up to the stage of multi-cellular lumen formation will also be presented.

2. Materials and Methods

2.1. Materials

Poly(ethylene terephthalate) 100- μm diameter monofilaments were used and fixed onto polycarbonate frame that fitted into traditional 6-well plates used in cell culture. Phosphate buffered saline solution (PBS 1X, pH 7.4) and Hank's balances salt solution (HBSS, H1387) were purchased from Sigma-Aldrich Inc. and used in these experiments.

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (cc-2519, Walkersville, MD USA). HUVECs were and cultured at 37°C and 5% CO₂ in modified M199 culture medium (M5017, Sigma-Aldrich). HUVECs between passages 2 and 6 were used in all experiments.

2.2. Three-dimensional Angiogenesis assays

Fibrin gels were firstly prepared to be used as the attachment bench, using 1 mL/well of fibrinogen solution (4.0 mg mL⁻¹) in HBSS that was mixed directly with 1 mL of a thrombin solution (2U mL⁻¹) in HBSS for the polymerization process of fibrinogen into fibrin (5 min at room temperature followed by 20 min at 37°C and 5 % CO₂). To evaluate cell adhesion on PET fibres, fibres-covered HUVEC was fixed after 4hrs and preceded for further nucleus staining process. Two different fibre-to-fibre distances (i.e., 100 and 200 micro meter) were tested in this study.

After 2 days of attachment process, fibres-covered cells were then transferred to the new well plate for the sandwich system for further angiogenesis assay. The numbers of cells as well as their connections were evaluated at day 2 and 4 of culture.

2.3. Angiogenesis images

Frame bearing fibres embedded in fibrin gel was firstly fixed with formaldehyde solution (3.75%, wt/v) for 20 min, followed by permeabilization process with Triton X-100 solution (0.5% v/v in PBS) for 5 min, and incubated 1 hr in a PBS solution containing 2% (wt/v) BSA.

Finally, the sample was stained with a mixture of TRITC-phalloidin (1:300 dilution, cat.#P1951, Sigma-Aldrich) and Hoechst (H33258, Sigma-Aldrich) (1:10,000 dilution). Pictures were taken either with epi-fluorescence or laser scanning confocal microscopy (LSCM).

3. Results and Discussions

Although endothelial cell adhesion on PET surface was known as negligible [5], the use of fibrin gel has been successfully increased cell adhesion. Data of cell adhesion after 4 hrs and following 2 days of culture allowing cells to proliferate and spread along the fibre axis, as presented on Table 1.

Table 1. Number of cell attachment on PET fibre surface

Adhesion Time	Fibre-to-fibre distances	
	0.1mm	0.2mm
4 hours	147.57	67.93
2 days	454.57	132.03
SD 4hrs	14.29	13.26
SD 2dys	60.97	23.91
SEM	34.07	
CD p<5%	74.98	

When HUVECs-covered fibres were sandwiched in the fibrin gel containing 100K of HUVECs, the cell-covered polymer fibres was able to dictate endothelial cell to develop cell-to-cell connection and tube-like structure (Figure 1). Further 4 days of culture, shows a better maturation of microvessel (Fig. 2a). Histological test on the microvessel prove the evidence of multi-cellular lumen (Fig. 2b).

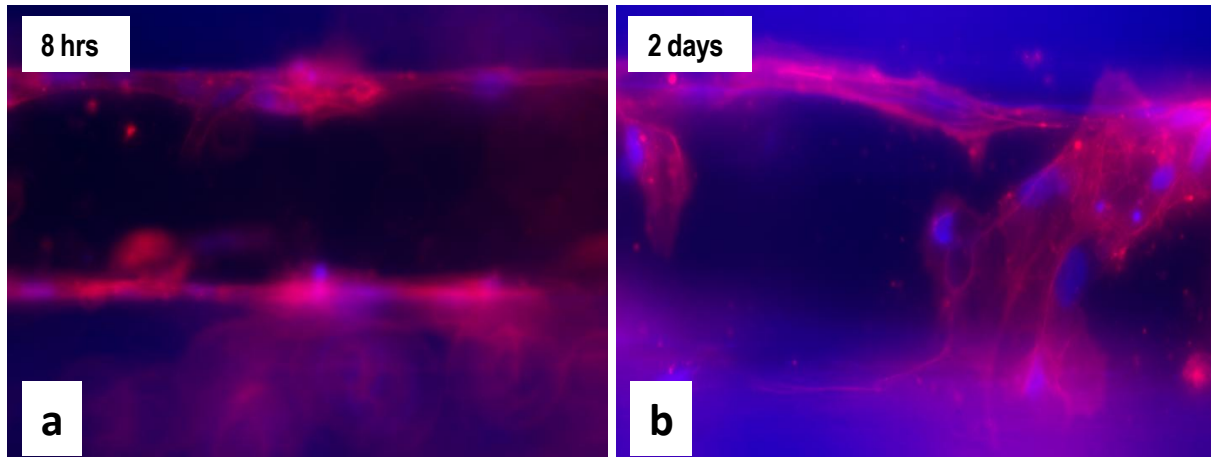


Figure 1. Cells-covered fibre dictates HUVECs undergoing angiogenesis. (A) Cell-to-cell connection. (B) Tube-like structure.

Based on those finding, it can be hypothesized that cell-covered fibres play a significant role as a bioactive sources and cellular guidance for the new cell inside the fibrin gel, which was also in the same agreement with other [6]. During the first 2 days of culture period, cell-covered fibre will migrate along the fibre axes and some are toward to the gel in order to build cell-to-cell connections.

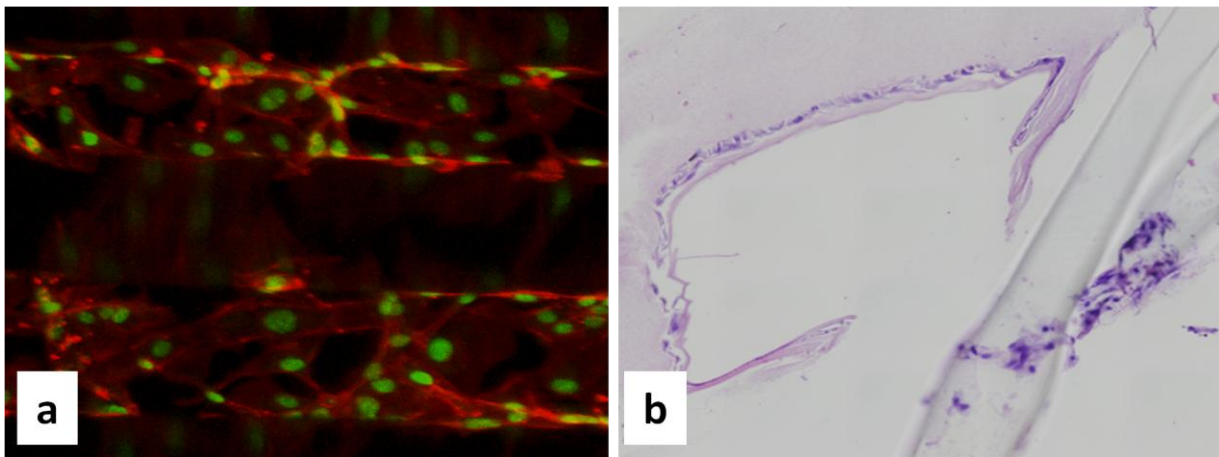


Figure 2. Assessment of microvessel formation. (A) Tube-like structure. (B) Multi-cellular lumen formation of HUVECs guided by polymer fibres.

4. Conclusions

This finding suggests that the direction of angiogenesis can be guided by the physical interactions involved between cells and polymer fibres to form a microvessel wall, allowing lumen formation. The system opens further potential applications in cardiovascular, cancer, and tissue engineering research. We hypothesize that the guidance of endothelial cell undergoing angiogenesis would subsequently facilitate the flow induction in order to vascularised engineering tissue construct.

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