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In vitro angiogenesis assay for the guidance of microvessel containing multi-cellular lumen formation

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Angiogenesis is a development process of new capillaries from the pre-existing blood microvessel. It involves multiple steps, including proliferation of endothelial cells (ECs), migration, alignment, sprouting, cell-to-cell connection, tube-like structure and lumen formation. One of the major problems faced by the angiogenesis researchers has been the difficulty of finding suitable in vitro assay to follow the morphological events during angiogenesis development. Morphological events of EC undergoing angiogenesis is of important knowledge for tissue engineering and biomedical societies. In this work we describe step-by-step angiogenesis process and morphological development of human umbilical vein endothelial cells (HUVECs) in the in vitro three-dimensional fibrin gel assays. Endothelial cell was firstly used to cover polymer fiber surface. When the fiber covered with cells was sandwiched between two fibrin gel containing cells, endothelial cell-covered fiber recruits cells inside the gel to form cell-to-cell connection and tube-like structure. Afterwards cells were degraded their fibrin matrix in order to form complex networks of microvessel containing multi-cellular lumen in between two adjacent fibres.

Keywords: Angiogenesis, Multi-cellular lumen, endothelial cells, cellular guidance.

1. INTRODUCTION

Angiogenesis, the growth and sprouting of new vessels from pre-existing blood vessel, is an essential process during normal development, such as organ growth and wound healing. It was also involved as a key role in the pathological disorders, including cancer growth and cardiovascular diseases. Recent advances in tissue engineering and cell imaging techniques allow the assessment of certain forms of culture in three-dimensional (3D) system.[1] Before that, cell culture systems used to conduct in vitro research have been predominantly of the two-dimensional (2D).[1,2]

In the 2D system, cells were exposed to the gravitational forces and to polarity of their environment. Numbers of observation taken from 3D systems show large discrepancies compared to the 2D [2]. For example, cancer and normal cells in 2D system exhibit similar morphologies and growth rates, while in 3D system both cell types showed different behaviours. This is accomplished in order to understand the of 3D systems parameters on cell response and to outline the importance of 3D systems to study cells in vitro.[1,3]

Several 3D systems of the in vitro and in vivo angiogenesis assays have been developed. Angiogenesis assay aimed to study the development of tube-like structure and multi-cellular lumens formation of endothelial cells.[4,5] Although some aspects of

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angiogenesis, including cell migration, proliferation and phenotype differentiation seem to be established, many studies fail to image and model the maturation of microvessel up to the stage of multi-cellular lumen development, as reported elsewhere.[5-7] In the research field of tissue engineering and cancer science, the imaging of a directional sprouting of tube-like structure and the assessment of multi-cellular lumen formation is of important.[3,7]

In the previous study, we have reported that sandwiched HUVEC-covered PET (poly-ethylene terephthalate) fibers in between two fibrin gel containing HUVECs allowed the guidance of microvessel formation.[8] The objective of this present study is to validate the imaging tools and assessment method of our 3D in vitro system for the guidance of oriented microvessels development. We hypothesize that an oriented tube-like structure and microvessels containing multi-cellular lumen of endothelial cells would facilitate the fluid flow for the supply of oxygen and nutrient to grow a tissue construct. Furthermore, having a system that can assist oriented microvessel development opens door for further study of tumour metastasis progression in cancer research.

2. EXPERIMENTAL DETAILS

2.1. Materials

Poly(ethylene terephthalate) (PET) monofilaments (Cat# ES305910, Goodfellow, Devon, PA) 100um in diameter were used and fixed onto polycarbonate (Boedeker Plastics, TX) frame that fitted into 6-well plates traditionally used in cell culture. Hank's balanced salt solution (HBSS, H6136) and albumin from bovine serum (BSA, A7906), cell culture medium M199 (M5017), endothelial cell growth supplement (ECGS, E2759), heparin (H1027), gelatine type B (G9391) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Plastic wares came from Fisher Scientific (Ottawa, ON, Canada). Trypsin-EDTA (25200-056), foetal bovine serum (FBS, 12483) and antibiotics (penicillin/streptomycin, 15140-122) were obtained from Invitrogen.

2.2. Cell Culture and 3D Angiogenesis Assay

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Walkersville, MD) and cultured at 37C and 5% CO2 in M199 containing 2.2 mg/mL sodium bicarbonate (Fisher, Fair Lawn, NJ), 90 ug/mL sodium heparin, 100 U/100 ug/mL pen/strep, 10% FBS, 2 mM L-glutamine (25030149; Invitrogen), and 15 ug/mL ECGS. HUVECs between passage 2-6 were used in this study.

Fibrin gels were prepared to be used as the attachment bench of HUVEC onto fibres using 1 mL/well of fibrinogen solution (4.0 mg/mL) in HBSS and supplemented with 350 KIU/mL of aprotinin and mixed with 1 mL of a thrombin solution (2U/mL in HBSS). After the polymerization 100K HUVECs then seeded directly to the fibre area then covered with 2mL of M199. This attachment process runs up to 2 days before transferred to the new well. For the evaluation of PET fibres' biocompatibility, fibres-covered HUVEC was fixed after 4hrs and preceded for further nucleus staining process. In this study, we tested two different fibre-to-fibre distances (i.e., 100 and 200 micro meter). After the attachment process (the first stage), frames bearing fibers were transferred to a new gel with the same composition as that described above and then covered with a second layer of fibrin that contained 100K HUVECs. After 2 and 4 days of sandwiched culture, the system were then fixed and evaluated.

2.3. Image and Images Processing

To observe endothelial cell morphology, samples were inspected daily under phase contrast microscopy. To prepare the fluorescence image, cell-seeded fibers were gently washed with PBS (3 times) and fixed in a formaldehyde solution (3.75%, wt/v) in PBS for 20 min then 3 times PBS wash. Cells adhered on fibers were permeabilized with a Triton X-100 solution for 5 min, after rinsing, samples were incubated for 1 h in a PBS solution containing 20% (wt/v) BSA. After that, samples were incubated in a solution containing a mixture of TRITC-phalloidin (1:300 dilution) and Hoechst 33258 (1:10,000 dilution) made in a blocking buffer solution containing 20% of BSA for 1 h at room temperature in the dark and rinsed three times with PBS.

The numbers of cell and cell-cell connections (capillary tube-like structure) were manually quantified after 2 and 4 days of culture by counting the numbers of sprouts and branch points. A sprout is an elongated structure where a connection starts, whereas branch point defined as areas where a single trunk structure gave rise to several divergent outgrowths.

2.4. Laser Scanning Confocal Microscope Images

To analyze microvessels between adjacent fibers, frames bearing fibers embedded in fibrin were incubated in a solution containing a mixture of TRITC-phalloidin (1:300 dilution) made in a blocking buffer solution containing BSA [20% (wt/v) in PBS] for 1 h at room temperature in the dark and rinsed three times with PBS. Finally, samples were incubated with SYTOX Green Nucleic Acid Stain (1:20,000 dilutions in PBS) for 20 min and washed three times with PBS.

Images of microvessels were taken with a Biological Laser Scanning Confocal Microscope (Olympus Fluoview FV300; Olympus Optical, Tokyo, Japan) equipped with an Olympus IX70 camera and recorded as high-resolution and as layer-by-layer files and a complete image reconstruction. Images were edited with the Image-Pro Plus Software to identify microvessels and lumen formation along fiber axis.

To assess the degradation of fibrin extracellular matrix by HUVECs, human fibrinogen conjugated with Alexa Fluor 546 (F-13192, Molecular Probes, Eugene, OR) was combined with unlabelled human fibrinogen at a mass ratio of 1:10 (conjugated to unconjugated fibrinogen) to produce the fibrin gel, in which the HUVEC-covered

fibres were sandwiched. In this experiment, HUVEC were labeled with the CellTracer CFSE staining (C34554, Molecular Probes, Eugene, OR) for 30 min prior to the experiment. The fibrin degradation by HUVEC could then be examined using confocal microscopy.

2.5. Histological Sections

Fibrin gels containing fibre holders were fixed overnight in 4% neutral buffered formalin. Fibres embedded in fibrin were gently removed from their frame, and processed for paraffin sections according to standard protocols.[9] Six-mm-thick sections were then prepared for hematoxylin and eosin staining (both from Sigma-Aldrich). Pictures were taken with the bright field mode of the BioImaging Navigator microscope (Olympus FSX100, Olympus Optical Co. Ltd., Tokyo, Japan).

3. RESULTS AND DISCUSSIONS

Although cell adhesion on PET surface fibre was considered as negligible, using our technique with fibrin gel, allows a better cell adhesion result (Figure 1). Cells start to attach on the PET fibre surface (see arrow, Fig. 1a) early after seeding, while after 4hrs, cell start to be elongated (see arrow, Fig 1b). Fibre separated with 100micrometers in distance, has a better cell attachment when compare to 200micrometer (Fig. 1c). HUVEC-covered PET fibres are expected to increase bioactivity of the polymer fibre when sandwiched in the 3D system, which presented in the further results.

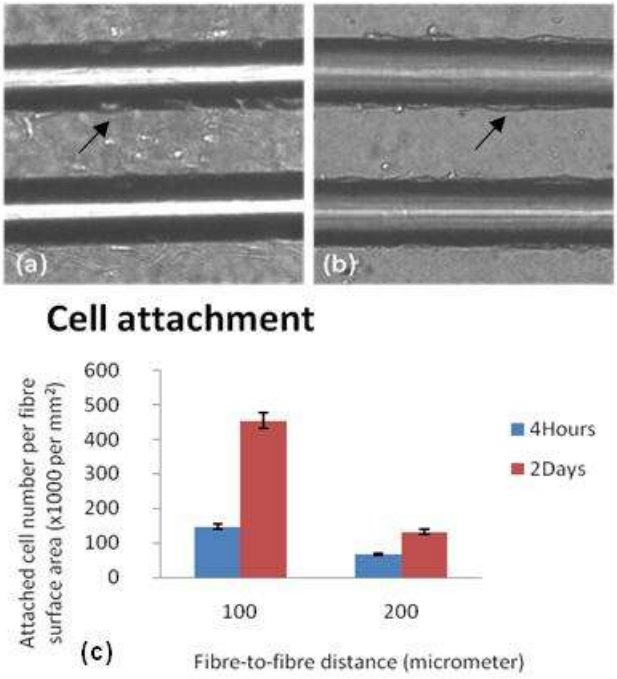

Figure 1. Endothelial cells adhesion on PET fibre surface

After 1 day of culture, some cells were able to build cell-to-cell connections along the fibre axis and even able to bridge between fibres, as shown on the phase contrast microscopy images (Fig. 2.a). Further 4 days of culture, the cell-to-cell connection appears homogenously in between two fibres (Fig. 2b). Higher magnification image (Fig. 2c) showed a tube-like structure of HUVEC and anastomosis development. Immunofluorescence images (Fig. 3) also confirmed the formation of cell-to-cell connections and tube-like structure development in between two fibre. The maturation process of the patterned microvessel also reveal when compare the microvessel image of two days (Fig. 3a) and four days of culture (Fig. 3b). This 3D system, in which HUVEC-covered fibres embedded in three-dimensional system containing HUVECs, allows a precise directional of microvessel and angiogenesis formation.

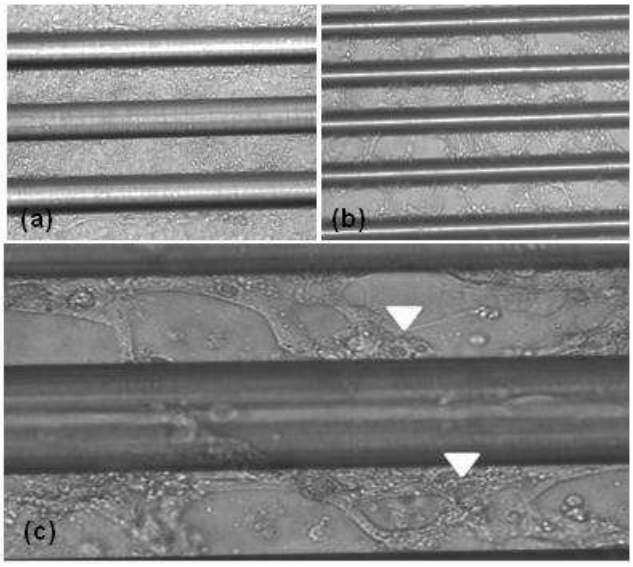

Figure 2. Tube-like structure of HUVEC

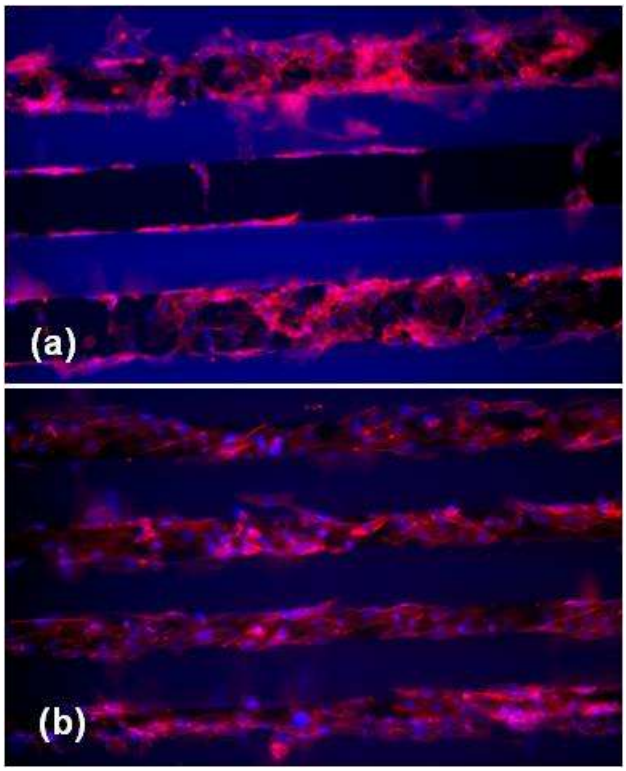

Figure 3. Oriented microvessels of ECs (a) after 2, and (b) 4 days of culture.

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Laser scanning confocal microscopy images of the experiment using fluorescence fibrin (Figures 4a-4c) also confirm the degradation fibrin extracellular matrix. In these images, extracellular matrix was made by a mixture of fibrin conjugated with Alexa Fluor 546 (red) and non-conjugated (regular) fibrinogen, while the nucleus of HUVECs were stained with Sytox Green. Further H&E staining of the histology sample (Fig. 4d) confirmed that the tube-like structure is multi-cellular and that the lumen is surrounded by multiple endothelial cells. The finding of a multi-cellular microvessel structure containing lumen in this study was in a good agreement with references.[10,11]

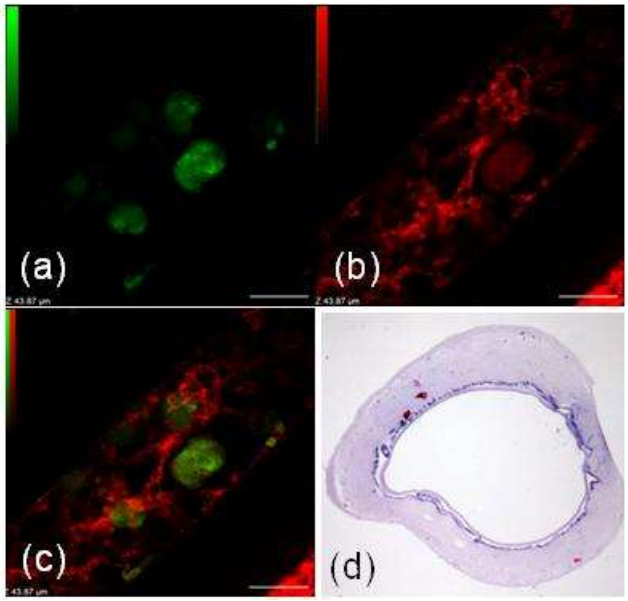

Figure 4. Assessment of the extracellular matrix degradation and multi-cellular lumen formation

4. CONCLUSIONS

Although Three-dimensional angiogenesis assay as well as imaging technique to study and assess particular morphological event during microvessel development is of important, which can advance our knowledge and understanding on step-by-step process of angiogenesis development in a 3D environment. Advanced microscope technology e.g., laser scanning confocal microscopy and traditional epi-fluorescence microscopy and histological analysis have been used to assess the step-by-step morphological events during microvessel and angiogenesis formation in a fibrin gel system.

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