Imaging tools for the analysis of microvessel and angiogenesis development in a tridimensional cell culture system

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Abstract—**This study presents the design and validation of imaging tools to access and analyses the development of microvessel and angiogenesis inside a tridimensional (3D) cell culture system. Angiogenesis is a key mechanism during normal development as well as in cancer cell growth. Phase contrast, fluorescence, and confocal microscopy were used to determine the cell attachment, tube-like structure formation and other cellular events inside our 3D system.**

Keywords-microvessel, angiogenesis; confocal microscopy; tridimensinal (3D) system; human umbilical vein endothelial (HUVEC) cell

I. INTRODUCTION

The importance of cell-morphological event during microvessel and angiogenesis development is coming into focus on the recent research of cancer, cardiovascular and tissue engineering. This study aims to present a 3D system for microvessel and angiogenesis guidance and method for quantitative analyses and evaluation.

Angiogenesis was defined as a development of new capillaries from pre-existing micrevessel [1]. It consists of a multiple steps, i.e. sprouting of human umbilical vein endothelial cell (HUVEC) from the parent vessel, followed by migration, proliferation, alignment, and the network of tube-like structure [2]. Several in vitro angiogenesis assays have been developed to study and follow the development of microvessel in a 3D system. Although some aspects of angiogenesis seem to be established among the scientific community, many studies fail to justify model and imaging tool to follow the cellular mechanism and morphological events involved in angiogenesis development [3,4].

In a previous study we have reported that polymer fibre i.e., poly-ethylene terephthalate (PET), sandwiched between two layer of fibrin gel containing HUVECs allowed the guidance of microvessel formation [5]. In this present study, we have optimized and validated our in 3D vitro model thus justify our evaluation methods of microvessel and angiogenesis formation. Phase contrast and fluorescence microscopy were used to collect data for quantitative evaluation of cell population and microvessel number, while confocal microscopy was used to determine the tube like structure inside our 3D system.

II. MATERIALS AND METHODS

A. Materials

Poly-(ethylene terephthalate) 100-µm diameter monofilaments (PET, ES305910, Good Fellow, Devon, USA) were used and fixed onto polycarbonate (Boedeker Plastics Inc., Texas, USA) frame that fitted into traditional 6-well plates used in cell culture.

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (cc-2519, Walkersville, MD USA). HUVECs were cultured at 37°C and 5% CO_2 in M199 culture medium (M5017, Sigma-Aldrich) containing 2.2 mg mL^{-1} sodium bicarbonate (Fisher, Fair Lawn, USA), 90 µg mL^{-1} sodium heparin (H1027, Sigma-Aldrich), 100 U/100 µg mL^{-1} penicillin/streptomycin (15140-122, Invitrogen Corporation, NY, Grand Island, USA), 10% fetal bovine serum (FBS, F1051, Sigma-Aldrich), 2 mM L-glutamine (25030149, Invitrogen Corporation), and 15 µg mL^{-1} endothelial growth factor supplement (ECGS, 356006, BD Biosciences, San Jose, CA, USA). HUVECs between passages 2 and 6 were used in all experiments.

B. 3D cell culture system

- Fibrin gels were prepared for cell attachment bench, (Fig. 1a). 1 mL of fibrinogen (2.0 mg mL^{-1}) in HBSS supplemented with 350 KIU mL^{-1} of aprotinin, was mixed directly with 1 mL of a thrombin solution (1U mL^{-1} in HBSS) for 5 min gelation at room temperature followed by 10-20 min at 37°C and 5 % CO_2).
- To count the number of cells attached on fibres surfaces, culture media were removed and the frames bearing fibres were incubated 1 hr with Hoechst 33258 (Sigma-Aldrich. Samples were observed under an inverted microscope (Eclipse TE 2000-S, Nikon Corp., Chiyoda-ku, Tokyo, Japan) using fluorescence and phase contrast imaging.

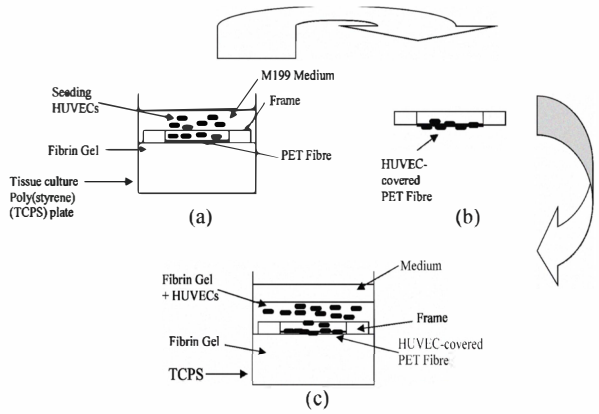

FIGURE I. 3D CULTURE SYSTEM

- After the attachment phase, fibres were transferred to a new gel (Fig. 1b) then covered with a second gel contained 100K HUVECs per mL (Fig. 1c).
- To analyze cells and connections, frame bearing fibres embedded in fibrin gel was fixed in a formaldehyde solution (3.75%, wt/v) for 20 min, permeabilized with a 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 then stained with a mixture of TRITC-phalloidin (1:300 dilution, cat. P1951, Sigma-Aldrich) and Hoechst 33258 (1:10,000 dilution).
- Images of microvessels were taken with a Confocal Laser Scanning Biological 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. A complete image reconstruction was made to illustrate microvessels in a 3D fashion.
- The histological sections, sample was fixed overnight then performed with hematoxylin (blue for nucleic) and eosin (purple for the matrix) staining

III. RESULTS AND DISCUSSIONS

Although cell adhesion on PET surface fibre is negligible [6], using fibrin gel, allow the attachment of HUVECs thus increase the biocompatibility of the polymer (Fig.1). After 4 hrs of experiment, cells start to attach on the fibre surface (Fig 1a), and following 2 days, cell start to elongated (Fig. 1b). Fluorescence dye of Hoechst33258 allows the evaluation to count the number of cell attachment which correspond to the nucleus of HUVECs (Fig. 1c). Further mix of Hoechst33258 with TRITC-Phalloidin for the actin filament of the cells allows the biocompatibility evaluation of PET fibre. Fig. 1d shows actin filament of HUVECs elongated along the fibre axis.

When cells covered fibres were sandwiched between two fibrin gel, HUVECs form tube-like cell connections followed by the sprouting and branching of the connections allowing the development of angiogenesis between adjacent fibres (Fig 3).

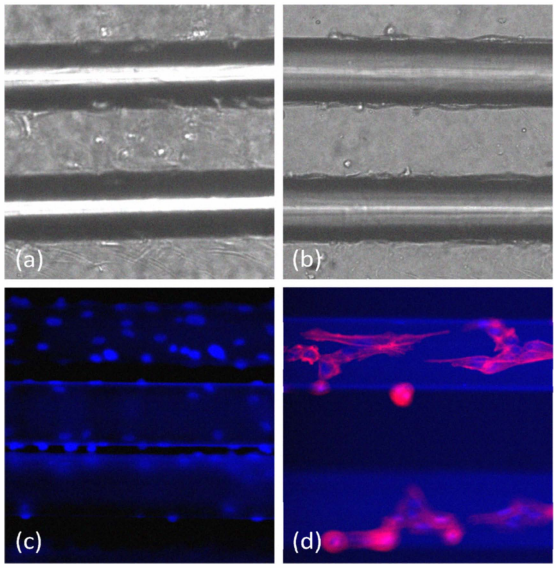

FIGURE II. IMAGE OF CELL ATTACHMENT

Confocal microscopy image confirm the formation of tube-like structure of microvascular network, as shown on complete reconstruction image (Fig. 3a) as well as layer by layer images (Fig 3b-c). Also, the H&E staining of the histology sample was showing that lumen was occurred inside the microvessels (Fig. 3d). Our finding of a multi-cellular microvessel containing lumen was in a good agreement with reference [3] and [4].

Using a confocal microscopy, we were able to determine the step-by-step process of angiogenesis development as follows:

- Stage 1: attached on the PET fibre and digest their surrounding matrix;
- Stage 2: committed cells proliferate, migrate, and communicate with each others in order to form tube-like connections;
- Stage 3: sprouting and lumen formation;
- Stage 4: development of a micro-vessel.

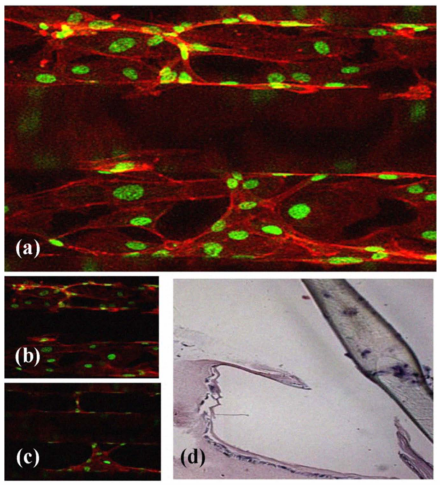

FIGURE III. CONFOCAL AND HISTOLOGY IMAGES

IV. CONCLUSION

Imaging tool and techniques to study cell and tissue development in a tridimensional system are poised to advance our understanding of angiogenesis development. Advanced microscopy technology e.g., confocal laser scanning microscopy has been used to determine the step-by-step morphological events during microvessel and angiogenesis formation in a fibrin gel system.

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