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The effects of co-culture with fibroblasts and angiogenic growth factors on microvascular maturation and multi-cellular lumen formation in HUVEC-oriented polymer fibre constructs

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ABSTRACT

In the present study, polymer monofilaments were embedded in fibrin seeded with human umbilical vein endothelial cells (HUVEC) to guide HUVEC attachment and migration in order to form oriented vessel-like structures between adjacent monofilaments. Histology and fluorescent fibrin experiments confirmed that microvessel-like structures, which were developing between polymer monofilaments embedded in fibrin, contained a lumen. The effect of human fibroblasts and growth factors (VEGF and bFGF) over the microvessel formation process was tested. The effects of VEGF and bFGF were dose-dependent. The effect of VEGF was optimum at the lower concentration tested (2 ng/mL), while that of bFGF was optimum at the higher tested concentration (20 ng/mL). Furthermore, the use of fibroblasts significantly improved the maturation of the microvessels compared to control and to samples cultured with VEGF and bFGF.

1. Introduction

Microvascularization is a process in which endothelial cells form microvessels. It is involved in the progression of cancerous cell masses [1–3]. Also, functional microvessels are necessary to supply nutrients and remove wastes in cell and/or tissue aggregates in order to improve tissue substitute survival and function [3,4], therefore justifying the development and application of methods to support microvessel formation.

Angiogenesis is a multistage process involving the sprouting of blood vessels from pre-existing vasculature [2,4]. Briefly, endothelial cells degrade the extracellular matrix followed by cell proliferation, migration, and cell–cell interactions leading to the formation of tube-like structures, and eventually to sprouting and lumen formation with adjacent vessels [4,5]. Several *in vitro* and *in vivo* angiogenesis assays have been developed to study and follow the development of sprouts and lumens and to validate the efficacy of both pro- and anti-angiogenic therapeutics [5,6]. *In vitro* assays include endothelial cells grown in different gels [6,7], microcarrier bead assays [8,9], and aortic rings embedded in gels [10–12], only to name a few. *In vivo* assays include the chick chorioallantoic membrane (CAM) [13], the Matrigel implant [14], and the retinal angiogenesis assay [15].

A number of reports have shown that pericytes [16] and smooth muscle cells [3] can maintain the patent tubular structure of blood vessels, and at the same time provide important factors to improve the maturation of microvessels [16,17]. Fibroblasts have been found to improve capillary sprout growth and stabilization in collagen and fibrin gels [16–18]. At some stage of the angiogenesis process, smooth muscle cells inhibit endothelial cell proliferation and new vessel formation, implying that mural cells interact with endothelial cells to stabilize newly formed capillaries [19].

On a different note, other studies reported that angiogenic growth factors, such as vascular endothelial growth factor (VEGF) [17,20] and basic fibroblast growth factor (bFGF) are essential for the induction of angiogenesis [21,22]. However, there are some contradictory data on how VEGF and bFGF concentrations influence cellular responses [12,17].

Although some aspects of angiogenesis, including endothelial cell migration, proliferation and phenotype differentiation seem to be established among the scientific community, many studies fail to

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model the maturation of microvessels up to the stage of lumen formation and even just to prove the existence of such multicellular lumen [17]. In addition, often microvessel networks are randomly distributed in a dispersing gel environment, and lack any patterning and specific orientation [22,23]. In tissue engineering applications and cancer research, the modeling and study of directional sprouting and microvessel formation is important [23,24]. Firstly, we believe that by orienting the development of microvessels, induction of fluid flow within the lumen of these microvessels will be facilitated [24]. Secondly, having a system in which microvessels can be patterned opens the door to new *in vitro* models of metastasis progression in cancer research [23–26] to better understand how biosignalling localization affects microvessel development in a 3D environment.

For these reasons, here we have further optimized and validated our *in vitro* model of microvessel patterning using polymer monofilaments embedded in fibrin. In a previous study, we have reported the development of an angiogenesis assay in which microvascularization process was modulated in a directional fashion [24]. It was demonstrated that polymer fibres deposited on top of a fibrin gel allowed cell attachment on these monofilaments. Furthermore, when HUVEC-covered fibres were subsequently sandwiched in fibrin, cells established connections with each others, thus leading to the formation of one microvessel between two adjacent fibres. Using this culture process, the aims of the present study were to investigate the effect of human fibroblasts and two angiogenic growth factors (i.e., vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) on microvessel maturation and to assess lumen formation.

2. Materials and methods

2.1. Materials

100-μm Diameter poly(ethylene terephthalate) monofilaments (PET, ES305910, Good Fellow, Devon, USA) were fixed onto polycarbonate (Boedeker Plastics Inc., Texas, USA) frames that fitted into traditional 6-well plates used in cell culture. The frames were 22.5 mm in diameter and 2.0 mm thick [24].

The phosphate buffered saline solution (PBS 1X, pH 7.4) used in these experiments was prepared from Milli-Q water and NaCl (0.137 M), KCl (0.003 M), Na_2HPO_4 (0.008 M), and KH_2PO_4 (0.002 M). This 150 mM solution was diluted in Milli-Q water to make a 10 mM solution. Hank's balances salt solution (HBSS, H1387) and albumin from bovine serum (BSA, CAS 9048-46-8) were purchased from Sigma–Aldrich Inc. (ON, Canada).

Vascular endothelial growth factor (VEGF) used here is a human recombinant $VEGF_{165}$ with a 38.2-kDa disulfide-linked homodimeric protein consisting of two 165 amino acid polypeptide chains. Basic fibroblast growth factor (bFGF) is a human recombinant with a 17.2-kDa protein consisting of 154 amino acid residues. $VEGF_{165}$ (cat.# 100-20) as well as bFGF (cat.# 100-18B) were purchased from PeproTech (PeproTech inc., NJ, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (C-12200, Heidelberg, Germany). HUVEC were cultured at 37 °C and 5% CO_2 in M199 culture medium (M5017, Sigma–Aldrich) supplemented with 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, Grand Island, NY, USA), 10% foetal 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). HUVEC between passages 2 and 6 were used in all experiments. Skin fibroblasts extracted from human foreskin and purchased from PromoCell (C-12350, Heidelberg, Germany) were cultured in M199 supplemented with 10% FBS and 100 U/100 μg mL^{-1} penicillin/streptomycin at 37 °C and 5% CO_2. Fibroblasts between passages 6 and 15 were used in all experiments.

2.3. Culture system and fibrin preparation

PET fibres were fixed on polycarbonate frames to be precisely distanced from each other [24]. Frames bearing fibres were cleaned in RBS detergent 35 (cat 27952, Pierce Biotechnology, Rockford, IL), sonicated for 15 min, rinsed with a flow of Milli-Q gradient water (Millipore Canada, Nepean, Canada) with a resistivity of 18.2 MΩ cm, and finally blow-dried with 0.2-μm filter-sterilized air. Frames bearing fibres were then immersed in 2 mL of sterile PBS containing 10% antibiotics (100 U/100 μg mL^{-1} penicillin/streptomycin) and exposed under UV light for 30 min, as the final sterilization step.

In 6-well plates, fibrin gels were prepared to be used as the attachment bench using 1 mL/well of fibrinogen solution (4.0 mg mL^{-1}) made in HBSS and supplemented with 350 KIU mL^{-1} of aprotinin. This solution was directly mixed with 1 mL of a thrombin solution (2 U 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% CO_2.

2.4. Cell adhesion and angiogenesis assay

After the polymerization process, sterile frames bearing the fibres were transferred and deposited on the top of a fibrin gel prepared as described in Section 2.3. Then, 100,000 HUVEC were directly seeded over it and cultures were run for 2 days to allow cell attachment. Two (2) mL of supplemented M199 medium were finally added to each well to cover the frames [24].

After the attachment phase, frames bearing fibres were transferred to a new fibrin gel with the same composition as described above, and then covered with a second layer of fibrin that contained 100,000 HUVEC per milliliter suspended in heparin-free M199 medium. The fibrinogen solution was allowed to clot for 5 min at room temperature and then, at 37 °C and 5% CO_2 for 30 min. After the polymerization process, 2 mL of M199 culture medium (with or without growth factors) were poured over it. Culture media were changed daily and the culture process was evaluated after 2 and 4 days. For the co-culture experiment, 300,000 of human foreskin fibroblasts were mixed directly with M199 culture media and added on the top of the fibrin. In another set of experiments, HUVEC were fed with M199 supplemented with either VEGF or bFGF (2, 5 or 20 ng/mL) with no fibroblasts.

2.5. Visualization of microvessels and lumen formation

To observe endothelial cell behaviour, pictures were daily taken with a phase contrast microscope. After 2 and 4 days, the fibrin gels containing the frames were fixed for further examination. For cell staining, cell-seeded fibres were gently washed with PBS (3 times) and fixed in a formaldehyde solution (3.75%, wt/v) in PBS for 20 min. Following three washes with PBS, cells adhered on fibres were permeabilized with a Triton X-100 solution (0.5% v/v in PBS) for 15 min. Samples were finally rinsed three times in PBS, incubated 1 h in a PBS solution containing 2% (wt/v) BSA, and rinsed three more times in PBS. Samples were incubated in a solution containing a mixture of rhodamine–phalloidin (1:300 dilution, R415, Molecular Probes, Eugene, OR) for F-actin labelling and Hoechst 33258 (1:10,000 dilution, cat. #B2883, Sigma–Aldrich) for nuclei staining, made in a blocking buffer solution containing BSA (2% (wt/v) in PBS) for 1 h at room temperature, in the dark. Finally, after three washes with PBS, samples were conserved with 3 mL per well of PBS.

Samples were imaged and recorded as high-resolution files (*.tif). To improve quality, images were then processed with Image-Pro-Plus Software. The number of cell–cell connections was manually quantified after 2 and 4 days of culture by counting the numbers of sprouts and branch points. Sprout is an elongated structure where a connection starts, whereas branch points are single trunk structures that give rise to two divergent outgrowths. The numbers of cells as well as cell–cell connections per fibre length are reported.

To assess the formation of multi-cellular lumen, 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, as in Section 2.3, in which the HUVEC-covered fibres were sandwiched. In this experiment, HUVEC were labelled 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.

In another series of experiments, fibres covered with unlabelled HUVEC were sandwiched between fluorescent fibrin (see the above paragraph for composition) and after polymerization, fibroblasts were subsequently added on the top of the fibrin gel. The cultures were carried out for a period of 4 days. Following fixation and permeabilization, as described above, cells were stained with Alexa Fluor 488 green phalloidin (1:300 dilution, A12379, Molecular Probes, Eugene, OR) for F-actin and Hoechst for nuclei. Layer-by-layer images were taken with a Confocal Laser Scanning Biological Microscope (Olympus Fluoview FV1000, Olympus Optical Co. Ltd., Tokyo, Japan).

2.6. Histological section

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 [27]. Six-μm-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