Polymer fibers as contact guidance to orient microvascularization in a 3D environment

Irza Sukmana,[1,2] Patrick Vermette[1,2]
[1]*Department of Chemical and Biotechnological Engineering, Laboratoire de Bioingénierie et de Biophysique de l'Université de Sherbrooke, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1K 2R1*
[2]*Research Centre on Aging, Institut universitaire de gériatrie de Sherbrooke, Sherbrooke, Québec, Canada, J1H 4C4*

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Abstract: We describe an *in vitro* culture process that uses 100-μm diameter poly(ethylene terephthalate) monofilaments as contact guidance of human umbilical vein endothelial cells (HUVECs) to orient the development of microvessels in a 3D environment. Untreated fibers, distanced either by 0.05, 0.1, 0.15, or 0.2 mm were first covered with HUVECs and then sandwiched between two layers of fibrin gel containing HUVECs. After 2 and 4 days of culture, cell connections and microvessels were evaluated. Cell connections formed massively along and in between adjacent fibers that were distanced by 0.05 and 0.1 mm, whereas with fibers separated by larger distances, connections were rare. After 4 days of culture, the optimum fiber-to-fiber distance to form microvessels was 0.1 mm. This study reveals that polymer fibers embedded in gel can be used as guides to direct the microvascularization process, with potential applications in cancer and cardiovascular research and tissue engineering. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 1587–1597, 2010

Key words: polyethylene terephthalate; fibers; cell adhesion; cell spreading; contact guidance; cell patterning; directional angiogenesis; microvessels

INTRODUCTION

One of the recent developments in the field of biotechnology is tissue engineering, which is the application of engineering principles and medical sciences to develop tissue substitutes that can be used to replace, repair, or regenerate damaged, injured, or missing body parts.[1–3] Although massive progress has been made, some major problems are still left to culture of human tissues. The lack of a sufficient supply of nutrients and oxygen to growing tissues *in vitro* and waste removal are two important factors that often lead to the failure of the culture process or even to that of implants.[3,4] One possible strategy for creating three-dimensional (3D) engineered tissue substitutes *in vitro* is to develop a scaffold allowing the guidance of cells to promote microvascularization in directional fashion.

Pre-vascularization of tissue-scaffold appears to be the most favorable and efficient approach to address the problem of tissue survival caused by a lack of oxygen supply. Pre-vascularization mainly involves the incorporation of endothelial cells or vascular-like structures into a scaffold and then the implantation of this scaffold at the desired anatomical site. This approach could favor a link between the existing vasculature from the host and the endothelialized tissue construct. Furthermore, it could accelerate the formation of functional microvessels in the interior of an implant.[5,6]

Angiogenesis is a key mechanism involved in the development of new blood vessels and wound healing.[7,8] Several assays have been described to grow networks of capillaries and to promote angiogenesis in a 3D environment, often composed of gel.[9–12] Although some steps of the angiogenesis process have been identified, the origin and mechanisms involved in lumen formation have been the subject of some debates.[13] The term lumen is sometimes used to describe features composed of cells forming circular structures in a nearly 2D plane rather than

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Correspondence to: P. Vermette; e-mail: Patrick.Vermette@USherbrooke.ca

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3D tube-like structures made of multiple and interconnected cells, as also suggested by others.[14]

Other approaches have been developed to modulate angiogenesis with the use of pre-vascularized scaffolds made of either natural fibers[15,16] or polymer fibers.[17] Some cells can orient and often migrate along the fiber axis. Cells can also bridge from one fiber to another if fibers are separated by distances small enough.[18–21] The fiber-to-fiber distance and the pattern by which fibers are presented to cells can be adjusted to modulate cell guidance.[19–21] Furthermore, as shown in a previous study, fibers precoated with cell-adhesive molecules, such as the RGD peptide, were used to guide cell responses.[21]

The objective of this study was to develop and validate a culture process that can be subsequently scaled to bioreactor cultures and that uses polymer fibers to guide the development of microvessels. We hypothesize that the orientation of microvessels would subsequently facilitate the flow induction within microvessel lumen for microvascularization and angiogenesis studies.

MATERIALS AND METHODS

Materials

Monofilaments (100-μm diameter) made of poly(ethylene terephthalate) (PET; ES305910, Goodfellow, Devon, PA) were used and fixed onto polycarbonate (Boedeker Plastics, TX) frames that fitted into wells of 6-well plates traditionally used in cell culture. The frames were 32.50 mm in diameter and 3.00 mm thick [Fig. 1(b)]. A scanning electron microscopy (SEM) picture of a PET fiber is presented as Supplementary Information, Figure i.

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 m*M* solution was diluted in Milli-Q water to make a 10 m*M* solution. Hank's balanced salt solution (HBSS; H1387) and albumin from bovine serum (BSA; CAS 9048-46-8) were purchased from Sigma-Aldrich. (St Louis, MO).

Cell culture

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

PET fibers and fibrin gel preparation

PET fibers were fixed on frames [Fig. 1(a,b)] to be precisely distanced from each other [Fig. 1(c)]. Frames bearing the fibers were cleaned in detergent (RBS Detergent 35; Pierce Biotechnology, Rockford, IL; cat 27952), 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 the fibers were then immersed in 2 mL sterile PBS containing 10% antibiotics (100 U/100 μg/mL penicillin/streptomycin) and exposed under a 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 (2.0 mg/mL) in HBSS and supplemented with 350 KIU/mL of aprotinin. This solution was mixed directly with 1 mL of a thrombin solution (1 U/mL in HBSS) for the polymerization process of fibrinogen into fibrin (5 min at room temperature followed by 10–20 min at 37°C and 5% CO_2).

Cell adhesion assay

After the polymerization process, sterile frames bearing the fibers were transferred to the top of this fibrin gel. Then, 100,000 cells were seeded directly over the frames bearing the fibers for cell adhesion. M199 medium (2 mL) was finally added to each well to cover the frames [refer Fig. 1(d)].

Cell adhesion was investigated after 4 h of incubation, as described elsewhere.[21–23] To count the number of cells attached on fiber surfaces, culture media were removed from the wells and the frames bearing fibers were incubated for 1 h with Hoechst 33258 (cat. 23491-45-4; Sigma-Aldrich) at a dilution of 1:10,000 in PBS containing 20% (wt/v) BSA.

Samples were observed under an inverted microscope (Eclipse TE 2000-S; Nikon, Chiyoda-ku, Tokyo, Japan) using fluorescence and phase contrast imaging. Images were taken with a 10X lens in conjunction with a digital CCD camera (Regita 1300R; QIMAGING, Burnaby, Canada) and an imaging software (SimplePCI; Compix, Minneapolis, MN). The numbers of nuclei per fiber, corresponding to the number of attached cells, were counted manually after 4 h and 48 h following cell adhesion.

Cell culture in 3D environment

After the adhesion phase, 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 50,000 HUVECs per mL suspended in heparin-free M199 medium. The fibrinogen solution was allowed to clot for 5 min at room temperature and then at

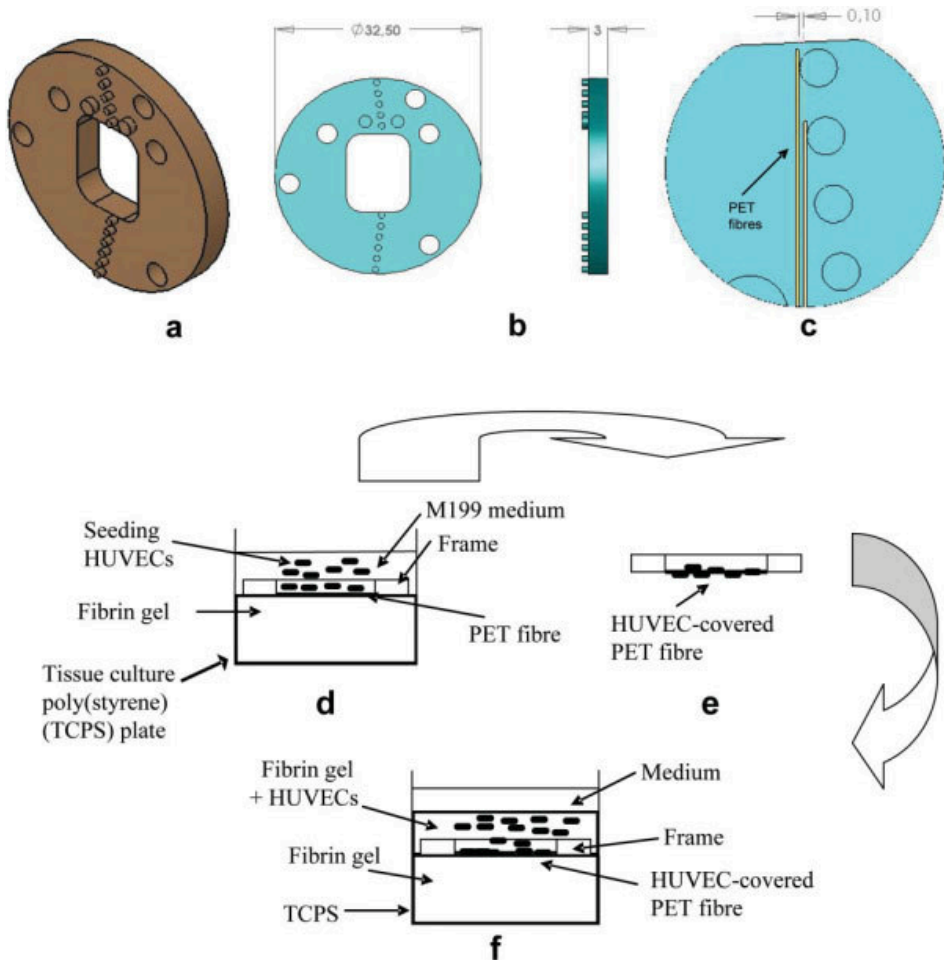

Figure 1. Schematic illustration of the *in vitro* method designed to guide and orient microvessel formation. Polycarbonate frames were produced by micromachining (a and b) to maintain fibers parallel to each other by well-defined distances (c). A layer of fibrin gel was prepared to serve as a support for endothelial cell (i.e., HUVEC) adhesion. Polycarbonate frames bearing PET fibers were placed over the gel, HUVECs were seeded directly over fibers, and M199 culture medium was added to cover the fibers (d). The culture period was allowed to proceed until HUVECs attached and covered fiber surfaces. When cells reached confluence, frames bearing fibers were transferred to a new plate for further culture (e). PET fibers covered with HUVECs were sandwiched between two fibrin gels and culture medium was added (f); only the upper gel contained HUVECs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

37°C and 5% CO_2 for 10–20 min. After the polymerization process, 2 mL of M199 culture medium was poured over it. Culture media were changed daily, and the culture process was evaluated after 2 and 4 days [Fig. 1(e,f)].

Visualization of microvessels

To observe endothelial cell behavior, samples were inspected daily under phase contrast microscopy and images were recorded. For cell staining, 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. Following three washes with PBS, cells adhered on fibers were permeabilized with a Triton X-100 solution (0.5% v/v in PBS) for 5 min. Samples were finally rinsed three times in PBS, incubated for 1 h in a PBS solution containing 20% (wt/v) BSA, and rinsed three times in PBS. Samples were incubated in a solution containing a mixture of TRITC-phalloidin (1:300 dilution; cat. P1951; Sigma-Aldrich) and Hoechst 33258 (1:10,000 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 and, finally, twice with Milli-Q water.

In addition, cells were stained with Dil-Ac-LDL (BT-902; Biomedical Technologies, Stoughton, MA) after 2 days of culture to analyze the formation of tube-like structures and to verify their phenotype.

The number of cell connections was 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 points were defined as areas where a single trunk structure gave rise to two divergent outgrowths.

Images and image processing

Fibers were imaged with an objective of 10X and recorded as high-resolution files (*.tif) to identify the effects of fiber-to-fiber distance over microvessel formation. An image processing software (Scion Image; Scion, Frederick, MD) and Picasa2 were used to improve image quality. These images were used to quantify the number of connections which originated from the newly formed tube-like connections along the fiber axis. The numbers of connections per fiber surface area as well as those per fiber length were reported.

Statistical analysis

The results were expressed as mean values ± standard deviations for each group of samples ($n = 12$), and after assessment of significant differences by one-way variance analysis (ANOVA), the level of significance was considered at a p value < 0.05 or < 0.01.

Confocal microscopy imaging of microvessels

To analyze microvessels between adjacent fibers, frames bearing fibers embedded in fibrin were gently washed with PBS (3 times) and fixed for 20 min in a formaldehyde solution (3.75%, wt/v, PBS). Following three washes with PBS, cells were permeabilized with a Triton X-100 solution (0.5% v/v in PBS) for 5 min. Samples were finally rinsed three times in PBS, incubated 1 h in a PBS solution containing 20% (wt/v) BSA, and rinsed three times in PBS. Samples 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 Milli-Q water. Finally, samples were incubated with SYTOX Green Nucleic Acid Stain (1:20,000 dilution in Milli-Q water; cat. S7020; Invitrogen) for 20 min and washed three times with Milli-Q water.

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. Images were edited with the Image-Pro Plus Software to identify microvessels and lumen formation along fiber axis.

RESULTS

Cell adhesion on untreated PET fibers

To better promote cell adhesion on untreated PET fibers, frames bearing fibers were placed on the top of a fibrin gel, and HUVECs were then seeded directly over it [Fig. 1(d)]. To evaluate cell adhesion and cell spreading, fiber frames were transferred to a new tissue culture poly(styrene) (TCPS) plate [Fig. 1(e)] to label cell nuclei. To induce the development of microvessels, HUVEC-covered PET fibers were then sandwiched in fibrin gel; the lower gel (i.e., that onto which fiber frames were deposited) was prepared without HUVECs whereas the upper fibrin gel (i.e., that covering fiber frames) was seeded with HUVECs [Fig. 1(f)].

HUVECs began to attach to untreated PET fiber surfaces after 4 h, as indicated by arrows in Figure 2(a). The culture was continued for a period of 2 days to allow cells to proliferate, migrate, and spread along the fiber and mostly at the interface between the fiber and the fibrin gel, as indicated by arrow heads in Figure 2(b).

Cell density was compared 4 h [Fig. 2(c)] and 2 days [Fig. 2(d)] following the initial incubation. To observe cell orientation and cell spreading along fiber surfaces following 2 days of culture, HUVECs were double-stained with TIRTC-phalloidin for actin filaments and with Hoechst 33258 for nuclei. The epifluorescence microscopy picture [Fig. 2(e)] revealed that actin filaments were well elongated along the fiber axis.

Cell adhesion on fibers is presented as the mean number of attached cells per fiber surface area (number of cells/mm^2) [Fig. 2(f)]. The number of anchored cells per fiber surface area increased as the fiber-to-fiber distance decreased; it also increased with time. After 4 h of incubation, the number of cells per fiber surface area was significantly ($p < 0.01$) higher with a fiber-to-fiber distance of 0.05 mm (179 ± 25 cells/mm^2) when compared to fiber-to-fiber distances of 0.1 mm (148 ± 14 cells/mm^2), 0.15 mm (99 ± 11 cells/mm^2), and 0.2 mm (68 ± 13 cells/mm^2). However, following 2 days of culture, the mean numbers of anchored cells per fiber surface area were on par with fibers distanced by 0.05 mm (473 ± 48 cells/mm^2) and 0.1 mm (455 ± 61 cells/mm^2) whereas the mean numbers of cells per surface area for fibers distanced either by 0.15 mm or 0.2 mm were significantly ($p < 0.01$) lowered, with values of 257 ± 70 cells/mm^2 and 132 ± 24 cells/mm^2, respectively.

Effect of fiber-to-fiber distance over cell connections and microvessel formation

When HUVEC-covered fibers separated from each others by 0.05 mm were transferred to new TCPS plates to be sandwiched in fibrin gel, within 4 h, some HUVECs formed connection bridges between adjacent fibers; for the larger fiber-to-fiber distances tested here, cells were more elongated along the fibers (Fig. ii, Supplementary Information). HUVECs

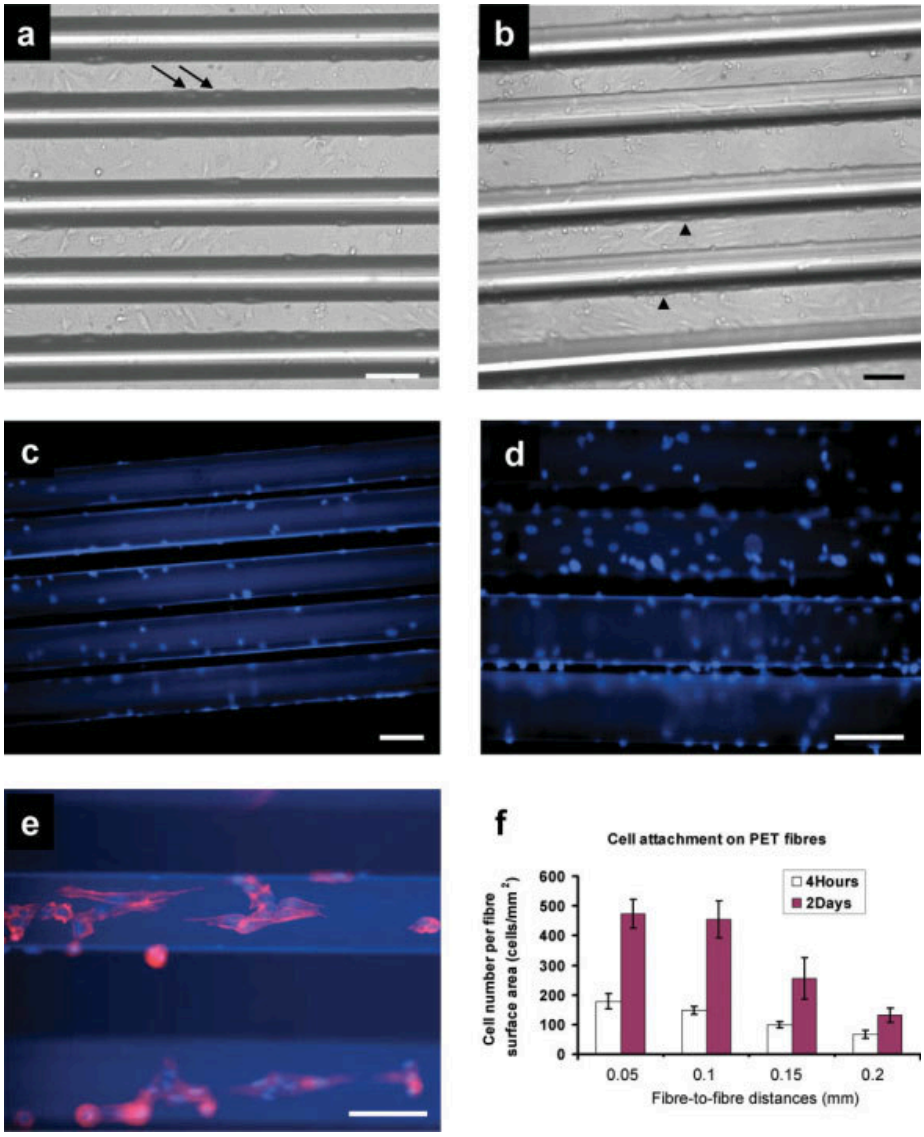

Figure 2. HUVECs attached to fibers after 4 h (Step 1d in Fig. 1), as shown by arrows (a). PET fibers were used to guide HUVEC proliferation along the fiber axis until 48 h, as shown by arrowheads (b). To count the number of attached cells, nuclei were stained with Hoechst after 4 h (c) and 48 h (d). Actin filaments and nuclei were stained with TIRTC-phalloidin and Hoechst, respectively (e). The number of attached cells on fiber surfaces increased as the fiber-to-fiber distance decreased and time progressed (f). Scale bars correspond to 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degraded fibrin along fibers. With fibers distanced by 0.1 mm, cell-to-cell connections attached to either one or two fibers adjacent from each other were formed (Fig. ii, Supplementary Information, and Fig. 3). When fibers were distanced by 0.15 or 0.2 mm, HUVECs needed more time to develop connections; even if some connections were found after 2 days, the density was much lower (Fig. 3).

Phase contrast microscopy pictures of HUVECs labeled with Dil-acetylated-LDL showed a good cell growth over fibers and stability of the cells phenotype over time (Fig. ii, Supplementary Information). Also, testing with Factor VIII at day 2 gives a positive labeling for endothelial cells (Fig. ii, Supplementary Information).

The mean numbers of cell connections between fibers distanced either by 0.05, 0.1, 0.15, or 0.2 mm were compared for periods of 2 and 4 days (Fig. 3 and Table I). The mean number of cell connections found between fibers distanced by 0.1 mm over 2 days (Fig. 3) was larger than those of fibers distanced either by 0.05, 0.15, or 0.2 mm. A similar trend was observed after 4 days (Fig. 3).

Cell connections along and between fibers as well as cell numbers per fiber surface area are presented in Table I as mean values ± standard deviations.

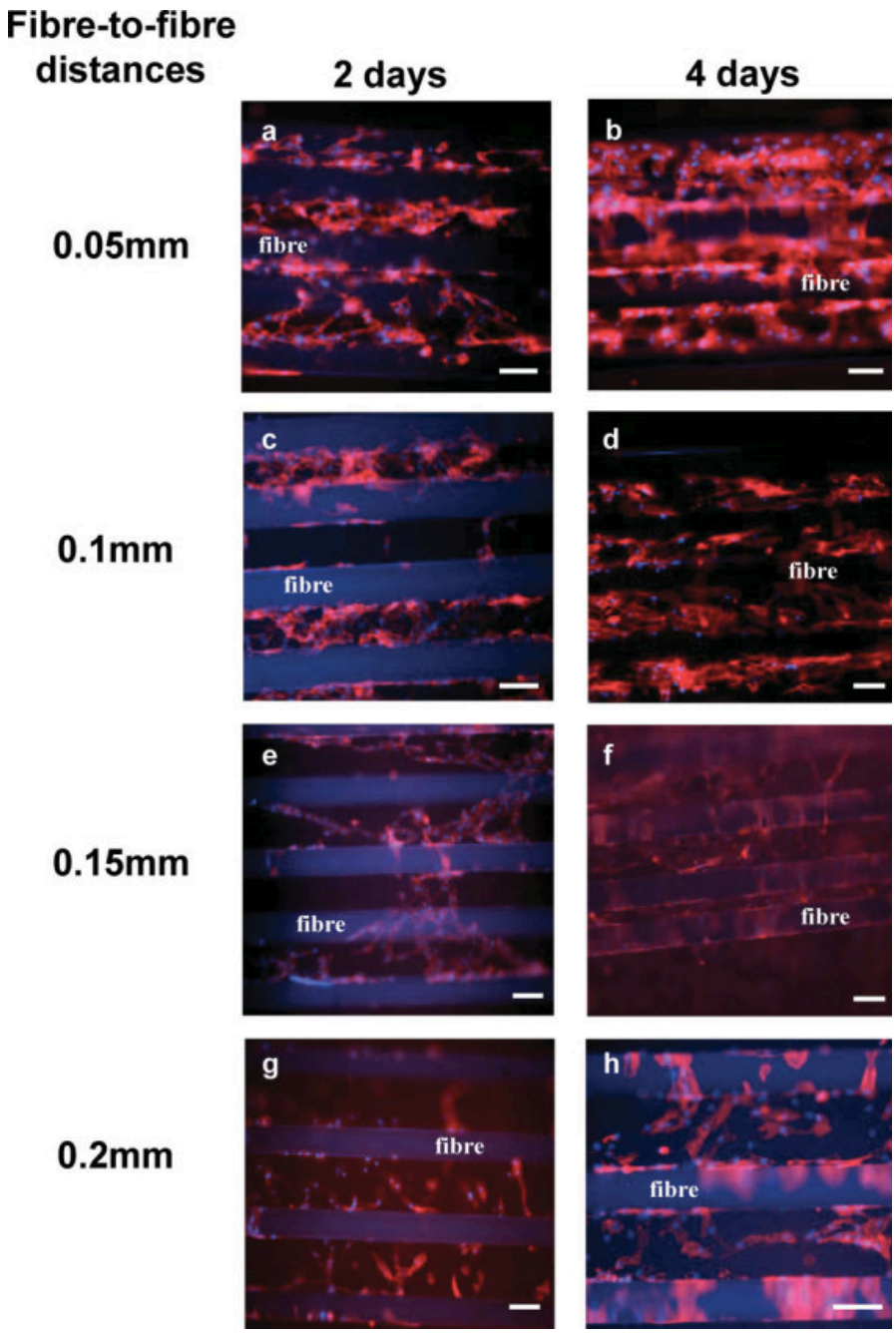

Figure 3. Effect of fiber-to-fiber distance over cell connections and microvessel development. HUVECs built cell connections, as observed by phase contrast microscopy, by day 2 (left-hand column) as well as by day 4 (right-hand column). Fibers were distanced by 0.05 mm (a and b), 0.1 mm (c and d), 0.15 mm (e and f), and 0.2 mm (g and h). Scale bars correspond to 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The numbers of connections per fiber length (number of connections/mm of fiber) after 2 days of culture were on par for fibers distanced by 0.05 mm (6.4 connections/mm) and 0.1 mm (7.0 connections/mm), whereas it significantly ($p < 0.01$) decreased with fibers distanced by 0.15 mm (2.6 connections/mm) and 0.2 mm (0.9 connections/mm). Following 4 days, the number of connections per fiber length for fibers distanced by 0.1 mm (12.1 connections/mm) was significantly ($p < 0.01$) larger than those of fibers distanced either by 0.05 mm (8.1 connections/mm), 0.15 mm (5.6 connections/mm), or 0.2 mm (2.1 connections/mm) (Table I).

The number of connections per fiber surface area (number of connections/mm^2) after 2 and 4 days of culture was significantly ($p < 0.01$) larger for the

TABLE I
Effects of Fibre-To-Fibre Distance on Cells

Parameters	Culture time (days)	Fibre-to-fibre distances (mm)				CD value	
		0.05	0.1	0.15	0.2	$p < 0.05$	$p < 0.01$
Number of cell connections per fibre length (connections/mm)	2	$6.4^{a} \pm 0.6$	$7.0^{a} \pm 1.8$	$2.6^{b} \pm 0.7$	$0.9^{c} \pm 0.3$	1.41	1.99
	4	$8.1^{b} \pm 2.3$	$12.1^{a} \pm 1.5$	$5.6^{c} \pm 1.7$	$2.1^{d} \pm 0.2$	1.91	2.69
Number of cell connections per fibre surface area (connections/mm^2)	2	$23.8^{b} \pm 2.1$	$28.2^{a} \pm 7.3$	$9.1^{c} \pm 3.7$	$2.4^{d} \pm 1.1$	5.84	8.25
	4	$25.8^{b} \pm 3.1$	$42.8^{a} \pm 11.3$	$14.5^{c} \pm 2.6$	$4.1^{d} \pm 1.3$	6.78	9.57
Number of cells per fibre surface area (cells/mm^2)	2	$338^{a} \pm 26$	$261^{b} \pm 18$	$167^{c} \pm 31$	$108^{d} \pm 32$	40.08	56.56
	4	$460^{a} \pm 157$	$332^{b} \pm 52$	$321^{b} \pm 60$	$133^{c} \pm 58$	125.67	177.34

Means in a row followed by different letters are significantly different.

0.1 mm fiber-to-fiber distance than those of fiber-to-fiber distances of 0.05, 0.15, and 0.2 mm (Table I).

The number of cells per fiber surface area increased when fiber-to-fiber distance decreased, as well as when the culture period increased. After 2 days of culture, the number of cells per fiber surface area for fibers separated by 0.05 mm (338 cells/mm^2) was significantly ($p < 0.01$) larger than those of fibers distanced by 0.1 mm (261 cells/mm^2), 0.15 mm (167 cells/mm^2), and 0.2 mm (108 cells/mm^2). After 4 days, the number of cells per fiber length was also significantly ($p < 0.05$) larger for a fiber-to-fiber distance of 0.05 mm (460 cells/mm^2), when compared with those of 0.2 mm (133 cells/mm^2) and 0.1 mm (332 cells/mm^2), in which the latter was on par with that of 0.15 mm (321 cells/mm^2) (Table I).

Microvessel formation

The confirmation of fully formed microvessels was verified by confocal microscopy for fibers distanced by 0.1 mm, from layer-by-layer confocal pictures [Fig. 4(a,e)], and from the complete image reconstruction. The complete image reconstruction [Fig. 4(f)] reveals the existence of a single lumen in between adjacent fibers. The higher magnification image [Fig. 4(g)] more clearly shows the presence of a lumen inside the network of cell connections (between upper and lower connections). The picture of microvessels taken using an inverted microscope is also shown as comparison [Fig. 4(h)].

In this study, we have successfully imaged microvessels with confocal microscopy, without the need to section the gel containing the cells and the fibers. In fact, histological analyses following embedding and sectioning were not possible because the fibers delaminated during sectioning, resulting in microvessel damage (data not shown).

DISCUSSION

Although cell adhesion on PET surface is limited or even negligible[21,24] because the surface is moderately wettable (contact angle of $\sim 75^{\circ}$),[25] some procedures have been successfully developed to improve PET fibers' biocompatibility.[21] The use of a fibrin gel to promote the ability of HUVECs to attach, proliferate, migrate, and spread along the PET fiber surface has been successfully demonstrated in the present study. Our findings are in good agreement with those of Khang et al.[18] that tested fibroblasts adhesion on PET fiber surfaces. Also, Hadjizadeh et al.[21] and Mirenghi et al.[24] show that it was possible to enhance human endothelial cell adhesion on plasma-modified and RGD-covered PET surfaces.

Comparing to a previous study by our group in which PET fibers were surface modified by plasma polymerization, the number of attached cells per fiber surface area obtained in the present study was larger. The better cell adhesion on untreated PET fibers observed with the method described here, as compared to other studies, can be explained in the following ways.

- The fibrin gel can stimulate cell responses because it contains several binding motifs such as the RGD binding peptides[21–23] and fibrin plasma proteins that interact with fibronectin to serve as a provisional extracellular matrix (ECM) for cell adhesion.[26,27]
- Fibers offer rigid interfaces to the cells, and as cells generally prefer rigid surfaces to soft ones, they will migrate to that interface.[28–30]

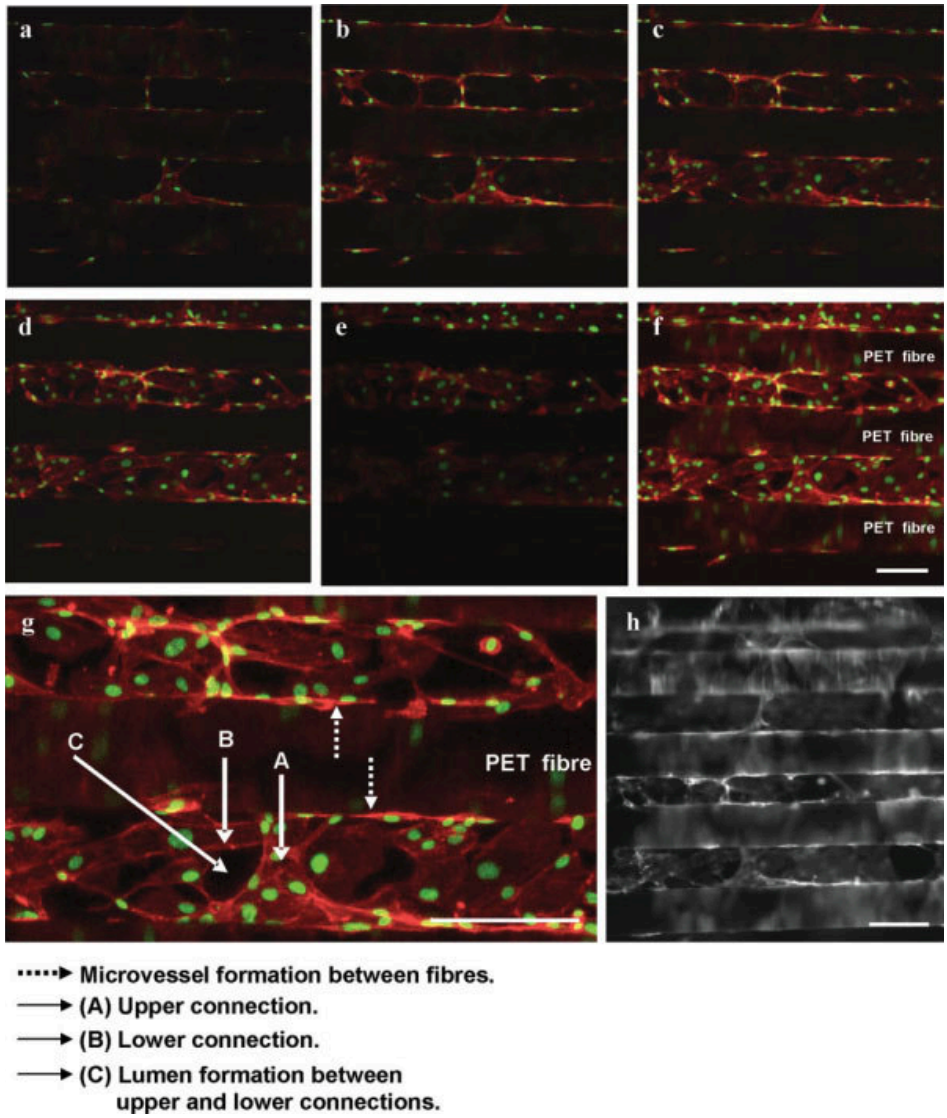

Figure 4. After 4 days of culture, cells were stained for actin filaments, using TRITC-phalloidin, and counterstained with SYTOX Green to highlight the position of the nuclei during confocal analyses. Confocal pictures were recorded layer-by-layer from the top to the bottom of samples (a–e). Complete image reconstructions are presented in (f) and (g) for a higher magnification of the lumen. A picture of microvessels taken using an inverted microscope is also shown as comparison (h). Scale bars correspond to 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

- The surface free energy provided by the bottom fibrin gel favors cell adhesion.[31]

Angiogenesis is referred to as the growth of new microvessels from pre-existing blood vessels. During angiogenesis, endothelial cells orient and organize themselves into tube-like structures with the presence of lumens, allowing connections of neighboring cells to subsequently form a network of tubes and initiate new blood vessels.[7,14,32] The angiogenesis process can be divided into four stages, which connect to the formation of microvessels[31]: Stage 1, cells degrade their surrounding matrix; Stage 2, committed cells proliferate, migrate, and communicate with each others to form tube-like connections; Stage 3, sprouting and lumen formation; Stage 4, development of a microvessel.

In our system, it can be hypothesized that microvessel formation occurred in the following steps. Early after HUVEC-covered fibers were embedded in the fibrin gel, spread cells detached from some areas of the fibers and migrate to the fibrin gel layer while other detached cells died. Within 4 h after the cell-covered fibers were sandwiched between fibrin gels, it is hypothesized that cells started to degrade fibrin along the fiber axis. After-

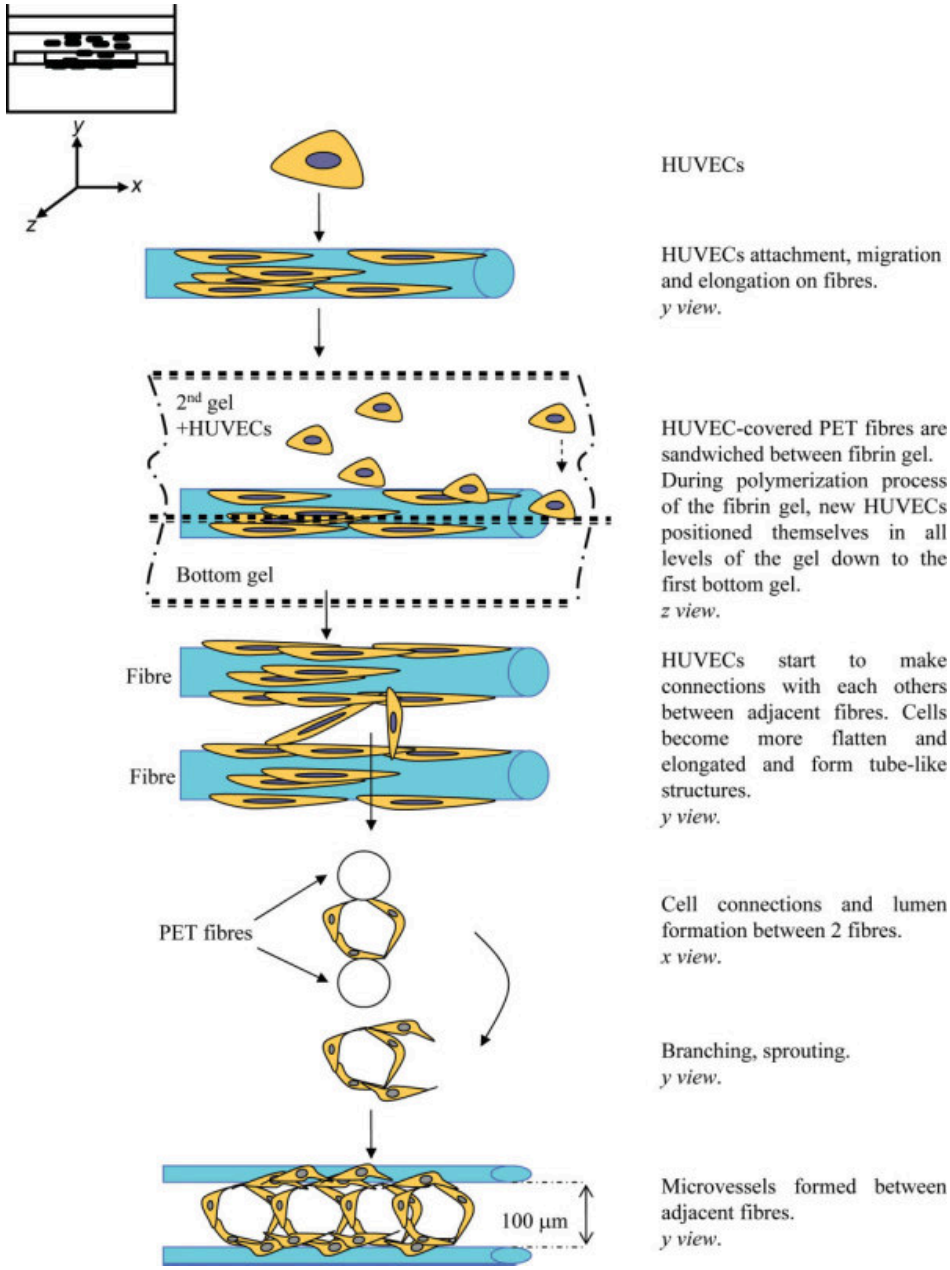

Figure 5. Schematic illustration of the microvascularization process. Adapted from Lutolf and Hubbell.[36] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

wards, cells were able to proliferate and migrate along fibers. Carrying the culture up to 24 h, cells on fibers and those inside fibrin gel interacted and built cell connections. During this phase, cell cytoskeleton shape became more flat and elongated, to form tube-like structures. The development of tube-like cell connections was followed by the sprouting and branching of cell connections until 2 days of culture, to form a lumen. The development of tube-like structures and lumen formation may be associated with a balance between cell migration activity and cell death, as also reported by other researchers.[33,34]

When the culture process was carried out up to 4 days, cells proliferated and migrated even more, as indicated by the increasing numbers of cell connections and cells per fiber surface area. Finally, cells formed one tube-like structure composed of multiple cells bridged between two adjacent fibers, as shown by confocal microscopy pictures (Fig. 4). This finding suggests that the direction of microvessel formation can be guided by the physical interac-

tions involved between cells and fibers to form a multicellular wall, allowing lumen formation. These microvessels that form between adjacent fibers distanced by 0.1 mm were associated by morphological events of the differentiation of endothelial cells into tube-like structures to subsequently create mature lumen, as also reported by others.[14,34,35] A complete schematic illustration of the proposed microvascularization development in the sandwich fibrin gel system containing PET fibers is shown in Figure 5.[36]

The fiber-to-fiber distance is an important parameter affecting the stimulation of cell adhesion as well as microvessel formation. In the adhesion phase, as compared to the larger distances tested here, smaller fiber-to-fiber distances give more possibility for HUVECs to sail over the gel to better attach to the rigid fiber surfaces. In this study, endothelial cells were placed at the interface between the rigid PET fibers and the soft fibrin gel. HUVECs tend to migrate and attach to the fibers; in particular, integrin cytoplasmic regions of the cells will convert mechanical forces into biochemical signaling in a process called durotaxis.[28,29]

When HUVEC-covered fibers separated by smaller distances (i.e., 0.05 and 0.1 mm) were sandwiched between two fibrin gels, within 4 h, cells migrated to form bridges between the fibers, stabilizing their cytoskeleton, and subsequently building connection strands. In the larger fiber-to-fiber distances tested in this study (i.e., 0.15 and 0.2 mm), cells required more time to form and stabilize their connection strands. The numbers of attached cells on the fibers and of cell connections play an important role in the development of lumen and microvessels, as also reported by others.[34,35]

CONCLUSIONS

This study reported the development and validation of a cell adhesion method that uses fibrin gel to increase HUVEC adhesion and spreading on untreated PET fibers. When fibers precovered with HUVECs were sandwiched between two fibrin gels, microvessel formation occurred along the fibers. The fiber-to-fiber distance was found to have a significant role in promoting cell adhesion, as well as cell connections between adjacent fibers, thus guiding microvessels formation along fibers in a 3D environment. Microvessels formed large tube-like structures (diameter of $\sim$100 μm) between adjacent fibers distanced by 0.1 mm after 4 days of culture. When fibers were separated by 0.05 mm, the distance was too small, so that connections lacked orientation and failed to form a large tube-like microvessel. In the larger fiber-to-fiber distances (i.e., 0.15 and 0.2 mm), cell connection strands were rare, and microvessels did not form between adjacent fibers. The requirements of this culture process were dictated by the possibility to scale the system to bioreactor cultures, which is the next step in our research program. This proof-of-concept study opens the door to other experiments in which we plan to study, using this culture process, the effects of cell number, fiber diameter, ECM density and type, cell types, and the presence of angiogenic growth factors on microvessel formation. Also, the functionality of these microvessels will be investigated.

This system can be applied to culture vascularized tissues such as in the development of a bioartificial pancreas device. These well-oriented microvessels can serve to supply nutrients to growing cells. If the fibers need to be removed from the constructed tissue before use or implantation, PET fibers can be replaced by biodegradable fibers.

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