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Development of fibrin gel-microgroove model for microvascularization by endothelial cells

Shaoyi Chen[1], Akihiro Morita[1], Irza Sukmana[2], Eijiro Maeda[3,*], Toshiro Ohashi[3]

[1]*Graduate School of Engineering, Hokkaido University, N13 W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan*
[2]*Department of Biomechanics and Biomedical Materials, Universiti Teknologi Malaysia, Johor Bahru, Malaysia (Present: Institute of Technology Bandung, Indonesia)*
[3]*Faculty of Engineering, Hokkaido University, N13 W8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan*

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Abstract This study was performed to develop a new experimental device with a fibrin gel-microgroove structure for study of microvascularization by endothelial cells (ECs). The effects of the width of microgrooves, initial cell seeding density and a supplementation of vascular endothelial growth factor (VEGF) on in vitro microvasculaization of ECs were examined. ECs were cultured in a fibrin gel formed on a polydimethylsiloxane microgroove substrate, with the microgroove width of 50, 100, 150 and 200 μm. ECs were elongated and sprouted within the gel in all the four types of microgrooves. In addition, multicellular network by connected cell branches were frequently observed in 100-μm microgrooves. Both high initial cell density and VEGF demonstrated significant promotional effects on morphology changes. The findings indicate that microgroove structure serves as a geometrical constraint for ECs, with a promotional effect on angiogenic responses of ECs, and thus, it can be used as an experimental model in the study of in vitro vascularization.

Keywords microvascularization, structure formation, microgroove width, cell seeding density, VEGF

Introduction

Vascularization, which is a phenomenon of *de novo* blood vessel formation of endothelial cells (ECs), plays an important role in many biological and pathological processes, such as embryogenesis, wound healing and tumor development [1]. From the findings of a number of *in vivo* and *in vitro* studies [2, 3], several factors, which affect new blood vessel formation, were suggested, such as topographic features, cell seeding density and vascular endothelial growth factor (VEGF).

Substrate topography and geometry were reported to help new blood vessel formation via cell-matrix mechanical interactions [4]. In a previous study, Sukmana and Vermette cultured ECs for several days with paralleled fibers, which was made from polyethylene terephthalate (PET) and sandwiched by two layers of fibrin gel, for stimulating microvascularization [5]. It was demonstrated that the PET fibers served as a geometrical guidance to restrict and align the cells towards the formation of tube-like structure between two adjacent fibers. It was also concluded that in the range of 50 μm to 200 μm, fibers spaced at a distance of 100 μm were an optimal condition for tube-like structure formation [5]. However, only one condition for fiber spacing could be examined in one device, which would impair the efficiency of examining further variations of fiber spacing distance as well as fiber diameter. Accordingly, the first purpose of the present study was to develop an alternative, new experimental model that can parallelize multiple experimental settings and examine these conditions in one device, and to examine if ECs are driven to form vascular-like structures in a manner depending on the size of a space between geometrical constraints.

For the rest of three major effectors of vascularization, high initial cell seeding density was reported to accelerate angiogenesis process [3]. However, since the results were obtained from different experiments and ECs were not of the same type, a systemic evaluation of the effects of cell seeding density on new blood vessel formation is still desirable.

VEGF, which is physiologically secreted by fibroblasts and inflammatory cells [6], has also been shown to influence

*E-mail: e.maeda@eng.hokudai.ac.jp (EM)

angiogenic process [7, 8]. It was reported that VEGF has the ability of changing EC shape during vascular lumen formation [9]. In another study of Sukmana and Vermette, it was demonstrated that VEGF has a promotional effect on tube-like structure formation between adjacent PET fibers and the effect was a dose-dependent with an optimal VEGF concentration of 2 ng/ml [10].

Therefore, the second purpose of this study was to examine if initial cell seeding density and VEGF also promote EC microvascularization in the newly developed microdevice.

Materials and Methods

Study design

The present study evaluated changes in morphology of ECs toward microvascularization (Fig. 1). ECs were initially in a round shape in a 3D culture environment as they were embedded within a fibrin gel in suspension. Unlike a 2D environment, ECs were expected to be elongated and branched out (sprouting cells). Cell branches were then connected to form a multicellular network, which would subsequently become a lumen of vascular-like microstructure. Thus, the efficiency of changes in cell morphology was evaluated with the different initial cell seeding densities as well as in the presence of VEGF.

Experimental model

PET fibers which had been used as a geometrical guidance to endothelial cells for microvascularization in previous studies [5, 10], were replaced with a micrometer-scale structure, microgrooves. Using MEMS techniques, microgrooves

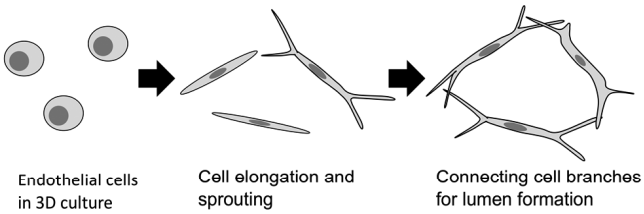

Fig. 1 Transition of endothelial cell morphology toward vascularization in a 3D environment.

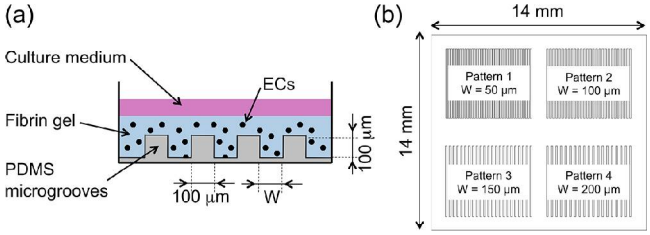

Fig. 2 (a) Schematic of newly developed experimental model consisting of microgrooved PDMS membrane at the bottom and fibrin gel containing ECs on top of the microgrooves. (b) Design of micropillar patterns.

with different sizes (groove width) can be patterned in a small area. Thus, multiple geometrical parameters can be examined in one device. For this, the experimental model was designed to consist of two components (Fig. 2(a)): microgroove structure patterned on a polydimethylsiloxane (PDMS) membrane and a fibrin gel embedded with ECs formed on top of the microgrooves. The microgrooves have a rectangular profile, with a groove width of 50, 100, 150 or 200 µm, a 100 µm spacing (ridge width) and the depth of 100 µm, and all the four patterns were fabricated in a square region in the PDMS membrane (Fig. 2(b)). The 100-µm-wide spacing mimicked the diameter of PET fibers used in the studies of Sukmana and Vermette [5, 10].

The microgrooves were fabricated through photolithography using a negative photoresist SU-8 3050 (MicroChem, USA) and softlithography using PDMS (Dow Corning, USA) on a circular coverslip (ϕ30, Matsunami, Japan). The PDMS microgroove membrane was then bound to the bottom of a bottom-less 35 mm cell culture dish (Iwaki, Japan).

The surface of microgrooves was coated with Pronectin F (Sanyo Kasei, Japan) to help cell adhesion. The fibrin gel embedded with ECs was formed on the microgrooves in the cell culture dish by incubating a mixture of 1 ml of fibrinogen solution (8 mg/ml), 1 ml of thrombin solution (2 U/ml) and a total of 0.5 ml of a mixture of cell suspension and culture medium adjusted to one of the following cell densities: 8×10^5 cells/ml (1×), 2× and 4×.

Cell culture

Bovine aortic endothelial cells (Cell Applications, USA) under passage 10 were cultured in a 3D environment within the fibrin gel for 4 days. A 2 ml of culture medium, consisting of Dulbecco's Modified Eagle Medium (Life Technologies, USA) supplemented with 10% fetal bovine serum (Life Technologies, USA), was placed on the top of the gel (Fig. 1(a)). To test effect of VEGF, $VEGF_{165}$ (Wako, Japan) was supplemented into culture medium at a concentration of 2 ng/ml.

Cell morphology analysis

After 4 days of incubation, BAECs were stained with rhodamine phalloidin (Life Technologies, USA) and Hoechst 33258 (Dojindo, Japan). A total of 10 fluorescence images of cells were obtained from randomly selected 10 regions from each microgroove pattern with a microscope (IX81, Olympus, Japan). Two parameters were selected for cell morphology analysis: the number of elongated cells, and the number of branches in sprouting cells (Fig. 3). These numbers were converted to the numbers of cells/structure per unit area.

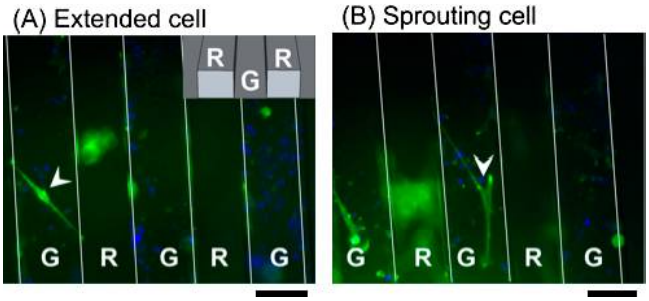

Fig. 3 Representative fluorescence images of (a) elongated cell (arrowhead), and (b) sprouting cell (arrowhead). G and R represent the groove region and the ridge region, respectively, as shown in an inserted schematic in (a). Ridge edges are outlined in white. Bars = 100 μm. Green: actin; blue, cell nuclei.

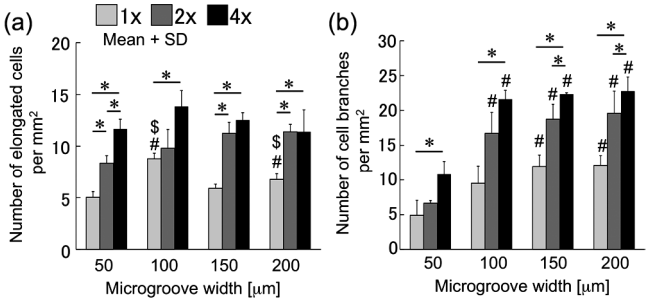

Fig. 4 The number of (a) elongated cells and (b) cell branches in each experimental condition. # $p < 0.05$ vs 50 μm; $ $p < 0.05$ vs 150 μm; * $p < 0.05$.

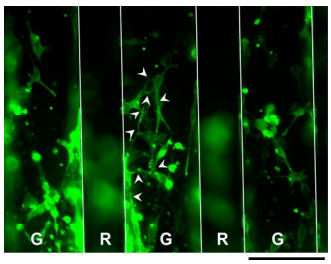

Fig. 5 Representative of the formation of multicellular network of connected cell branches in ECs cultured in 100-μm microgrooves. Arrows indicate the contacting points of cell branches. G and R present the groove region and the ridge region, respectively.

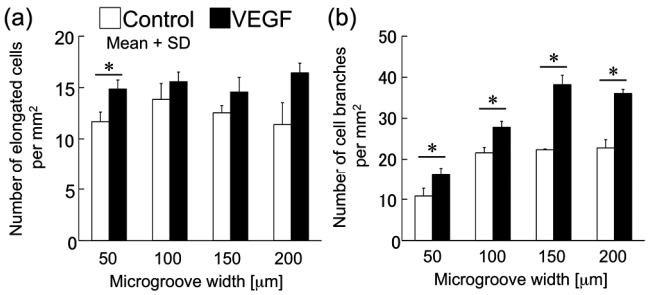

Fig. 6 Effect of VEGF supplementation on the formation of (a) cord-like cell structures, (b) sprouting structures and (c) cell circle structures. n = 3. * $p < 0.05$.

Statistical analysis

Experiments for examining effects of initial cell density and effects of VEGF were repeated three times. Differences in the numbers among the four microgroove widths and initial cell densities were compared with Tukey post hoc test following one-way ANOVA, and those induced by VEGF supplementation were examined with Student's t-tests. Statistical significance was set at $p < 0.05$.

Results

Effect of microgroove width

In the case of 1× cell density, the number of elongated cells was not systematically different, but the numbers in 50- and 150-μm microgrooves were significantly smaller than those in 100- and 200-μm microgrooves (Fig. 4(a)). On the other hand, the numbers of cell branches in 150- and 200-μm microgrooves were significantly higher than that in 50-μm microgrooves (Fig. 4(b)). In the cases of 2× and 4× cell densities, the numbers of elongated cells were not significantly changed among the four types of microgrooves. On the other hand, the trend in the number of cell branches in 2× and 4× cell densities was essentially similar with 1× cell density.

Effect of initial cell seeding density

There was a general trend that higher cell density resulted in larger number of angiogenic cell morphologies (Fig. 4). For elongated cells, the number in 1× density was significantly lower than that in 2× and 4× densities in all the four patterns of microgrooves, except for 100-μm microgrooves. For the number of cell branches, the trend was almost the same with the number of elongated cells; 4× density resulted in significantly higher number of cell branches than 1× density in all the four patterns of microgrooves. In addition, significant differences were also observed between 2× and 4× densities in 150- and 200-μm microgrooves. Furthermore, connections of cell branches, forming multicellular networks, were frequently observed in 100-μm microgrooves (Fig. 5).

Effect of VEGF supplementation

Effects of VEGF on microvascularization were tested at 4× cell seeding density (Fig. 6). Effects of VEGF on cell elongation were limited; a significant difference between non-treated control cells and VEGF-treated cells was only observed in 50-μm microgrooves. By contrast, cell branches were significantly increased from the levels of non-treated control cells in all the four patterns of microgrooves. In addition, the degree of the increase was the largest in 150-μm microgrooves.

Discussion

We have developed a new experimental system to investigate *in vitro* micro-vascularization of ECs, and examined if EC behaviors were regulated by 1) the size of a space between geometrical constraints (i.e. the width of microgrooves), 2) initial cell seeding density and 3) the supplementation of VEGF. It was clearly demonstrated that ECs changed their morphology to an elongated shape, followed by sprouting to form cell branches and forming multicellular network via connected cell branches, during the 4-day incubation period in the fibrin gel on microgrooves. Because no such changes in cell morphology were observed in ECs just cultured within a fibrin gel (data not shown), the elongation and sprouting of ECs were driven by the presence of microgrooves. The formation of multicellular network by cell branches was also reported when ECs were cultured in a fibrin gel, with fibroblasts cultured on the gel [11], possibly due to paracrine effects of growth factors, such as VEGF and basic fibroblast growth factor, synthesized from fibroblasts. Accordingly, microgroove structure serves as a geometrical constraint with a promotional effect on angiogenic responses of ECs.

It seems that effects of microgroove width on cell elongation were limited; significant differences were only observed in the case of 1× cell density (Fig. 4). By contrast, effects of microgroove width were more apparent in cell sprouting; a larger number of cell branches were produced in wider microgrooves. Moreover, in 100-μm microgrooves, it was frequently observed that cell branches connected with each other and formed networks. As such networks would subsequently lead to the formation of lumens in a vascularization process, the present finding that the formation of multicellular network was predominant in 100-μm microgrooves may suggest that 100-μm microgrooves were a preferred condition among the four types, which is consistent with the findings of previous studies [5, 10]. Therefore, this agreement proves that the microgroove structure can be an alternative experimental model to polymer fibers in the study of microvascularization.

The presence of microgrooves may not serve just as geometrical constraints. It has been reported that cells can sense the size of their 3D culture environment (i.e. the thickness of matrix gel) [12], possibly because of differences in apparent stiffness. In the present study, the microgroove ridges sectioned a continuous 3D space of fibrin gel into a series of narrow spaces with the width of 50, 100, 150 or 200 μm, creating 3D culture environments with different apparent stiffness. Thus, endothelial cells in fibrin gel might also sense differences in apparent stiffness of fibrin gel among the four types of microgrooves, and alter the way they adapt to their environments. In addition, difference in apparent stiffness of gel between 50 and 100 μm microgrooves might be more profound compared to differences between lager microgrooves. Thus, the cells could not sense the difference in the stiffness between 150 and 200 μm microgrooves, so that cell branches are almost the same in these large microgrooves.

Higher cell density was found to promote angiogenic morphological changes of ECs. It was reported that cell-cell recognition and connection were needed during lumen formation [13], which are possibly tight junctions [14]. Thus, it seems highly likely that cells in an environment of a high cell density have a greater chance to recognize and connect with other cells, further enhancing angiogenic responses. In addition, the promotional effects of VEGF on cell sprouting were consistent with past study [15]. Further experiment is needed to confirm that a co-culture of ECs with fibroblasts also has the same effects on angiogenic responses of ECs, through the secretion and paracrine effects of VEGF by fibroblasts.

Conclusions

The proposed fibrin gel-microgroove model can be used for study of *in vitro* maicrovascularization by endothelial cells. Microgrooves with the width of 100 μm were preferred geometrical structure for microvascularization. In addition, both higher initial cell seeding density and VEGF supplementation could promote the formation of angiogenic morphologies of endothelial cells.

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