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THREE-DIMENSIONAL ADVANCE DYNAMIC CULTURE SYSTEM PROMOTES MICROVESSEL DEVELOPMENT FROM CULTURED ENDOTHELIAL CELLS IN VITRO

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ABSTRACT

The present study describes a dynamic system that can be used in a three- dimensional (3D) in vitro cell culture environment which promotes new microvessel formation. Human umbilical vein endothelial cells (HUVECs) were used in this study to form the inner lining of the microvessel, guided by the dynamic flow produced by our system within a 3D matrix made from fibrin. A 2D environment was used as a comparison. The dynamic flow was set to produce 5, 10, 15 or 20 dynes/cm2 shear stress to the cells in culture. Cultured HUVECs were observed for the increase in cell numbers and formation of microvessels. Cells cultured after 2 days demonstrated an increase in cell numbers when subjected to 10dynes/cm^2 or more. By day 4, cells appeared to have altered morphologies and were oriented towards the direction of fluid flow. From the fluorescence images observed, it became apparent that there were microvascular channels forming in the 3D cultures. Our dynamic flow system appears to influence endothelial cells to promote microvascular formations in a 3D environment.

Keywords*:* dynamic cell culture, 3D system, human umbilical vein endothelial cells, microvessels, laminar shear stress.

INTRODUCTION

Microvascularization is a process formation of microvessels from endothelial cells (ECs) proliferation and migration. It is an essential fundamental process in the development of new blood vessel, wound healing, ovulation and embryonic development [1-2]. The mature and functionalized microvessels are necessary to supply nutrients and remove waste in cell or tissue in order to obtain a good tissue substitute [3-4]. A possible approach for creating mature microvessel in vitro is to culture the three-dimensional (3D) structures under dynamic environment, mimicking the in vivo conditions.

Numerous studies have been done previously to develop microvessel in vitro. Nakatsu *et al.* [5] developed a 3D model that promote sprouting, lumen formation and long-term stability microvessel. Park and his group [6] reported the formation of mature microvascular networks within 3D microfluidic fibrin gel scaffolds depend on the gel concentration and composition. Other studies also revealed that ECs culture in 3D environment were induced to proliferate, migrate, sprout, and made of multiple 3D tube-like structure [7-8]. However, these studies only limit on static environment, while the in vivo microenvironment was in dynamic.

A number of reports have shown that shear stress due to dynamic flow changed the orientation of cells in the direction of flow [9-10] and elongated more after few days [11-12]. Endothelial cells cultured under shear stress also have been proven to increase the cell proliferation and migration rate [13-14]. Recent in vitro studies also identified that shear stress increased the number of cell-cell contacts [15] and induced capillary-like construct [16-17]. However, those studies were only dealing in 2D construct which not represent real biological environment and limit the formation of mature and functionalized microvessels.

Here, we developed a 3D dynamic culture system to induce cells-cells connections and promote the development of microvessel formation. We hypothesized that the endothelial cells will grow in faster growth rate and form more mature microvessel under 3D environment and dynamic conditions as compare with 2D and static environment.

MATERIALS AND METHODS

Cells and materials

Human umbilical vein endothelial cells (HUVECs) between passages 2 and 6 were used in all experiments and purchased from Cambrex (cc-2519, Walkersville, MD USA). HUVECs were prepared for experiment by following the procedures described in Sukmana's work [8]. Acrylic (Boedeker Plastic, TX) flow culture chamber (FCC) fabricated in-house [Figure-1 (a)]. This chamber was used to culture the cells in 2D and 3D with dynamic flow condition.

Fibrin gel preparation

Fibrinogen solution (F8630-10G, Sigma Aldrich, USA) and thrombin solution (T4648-1KU, Sigma Aldrich, USA) were prepared in HBSS buffer. Solutions were heated at 37 ^oC for at least 20 min. In 12-multiwell plates, the suspension HUVECs was mixed directly with 250uL/well of thrombin solution (1U mL^{-1} in HBSS). This mix was placed either in the flow culture chamber or in a 12-multiwell plate for control experiments. 250 uL/well of fibrinogen (2 mg mL^{-1} in HBSS) was then added to the HUVEC-thrombin solution, to obtain a transparent and

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homogeneous fibrin gel. The final cell concentration was 3×10^4 HUVECs within a 20mm x 20mm x 2mm fibrin gel made in the flow culture chamber (FCC) (Figure-1(b)) and controls consisted of a 2-mm thick fibrin gels with cells. The fibrin gel was placed for 5 min at room temperature followed by 10-20 min at 37 ºC and 5% carbon dioxide (CO_2) for the polymerization process. Once the fibrin-HUVECs solution was polymerized, it was fed with 500uL M199 and incubated overnight for cell adhesion.

Flow culture chamber system

After the cells were already attached, the flow culture chamber was closed and connected to the dynamic culture system for the duration of 2 and 4 days of culture inside the 37 ºC and 5% CO_2 incubator for culturing environment control. The culture system condition was tested at four different flow rates to investigate the effect of different shear stress on HUVECs. A schematic design of the flow culture system and the picture during the experiment were presented on Figure-1 (c) and 1 (d) respectively.

Fluid shear stress was applied using flow culture chamber (FCC) system. The FCC was an in vitro model that simulates in vivo fluid shear stresses on various cell types exposed to dynamic fluid flow in their physiological environment. This device exposes cultured EC to a defined level of wall shear stress generated by the fluid flow. In this study, the FCC was placed in a closed series with a medium reservoir and peristaltic pump. The reservoir was filled with 125mL of M199 that re-circulated independently for each sample through the system. A peristaltic pump was used to positively displace the medium through the dampener, resulting in a steady flow of medium into the flow block. Using this system, we applied a steady laminar shear stress of 5, 10, 15 and 20 dyne/cm^2 for 2 and 4 days.

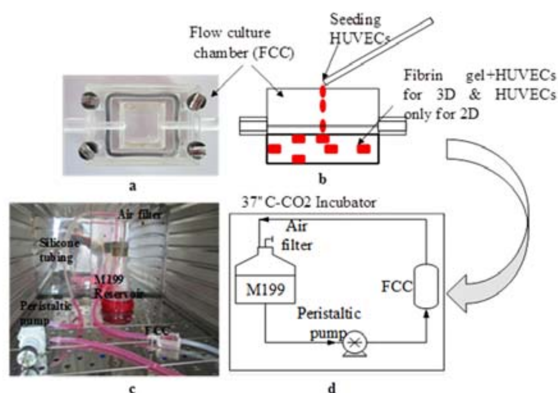

Figure-1. Acrylic flow culture chamber (FCC) (a). HUVECs were seeded directly in fibrin gel for 3D or on FCC surface without fibrin for 2D environment (b). After HUVECs attached FCC was connected to dynamic system (c), consists of a power supply, custom-made flow culture chamber and a micro-peristaltic pump connected to a bottle containing culture medium and air filter using silicone tubing was placed within the incubator (d).

Visualization of microvessels and lumen formation

During the experiment, endothelial cell behavior was daily accessed and pictures were taken with a phase contrast microscope. After the end of experiment of 2 and 4 days, the fibrin gels were fixed and stained following the procedures of Sukmana's work [8]. Finally, after three times washing with PBS, samples were conserved with 3 ml per well of PBS. Samples were imaged using (Axio Vert. A1, Zeis) and recorded as high-resolution files (*. tif). The images were then processed with Image-Pro-Plus Software to improve the quality. 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. The cell numbers were counted automatically using automatic cell counter (TC20, BioRad).

Imaging and image processing

The microvessel formation was imaged with an objective 10X and recorded as high-resolution files (*.tif) to identify the effects of shear stress over microvessel formation. Image processing software (Adobe Photoshop CC 14.0) was used to improve image quality. These images were used to observe the formation of lumen (tube-like connection).

Statistical analysis

The results were expressed as mean values ± standard deviations for each group of samples (n = 12). Data were statistically analyzed by using the analysis of one-way variance (ANOVA), with the level of significance was considered at a p value < 0.05.

RESULTS

Cells viability and proliferation

In order to investigate the cell proliferation, HUVEC were trysipnized and the number of cells were counted automatically using automatic cell counter (TC20, BioRad). The sample was prepared 3 times for each culturing environment and then analyzed using ANOVA. Cells proliferations over surface area are presented as mean values ± standard deviation in Table-1 for flow culture chamber.

The mean number of cells between two-dimensional, three-dimensional, static and dynamic environment were compared for the culture periods of 2 and 4 days. The mean numbers of cell found in three-dimensional with 15 dynes/cm^2 shear stress over 2 days (Figure-2) was larger than other cultural environments. A similar trend was observed after 4 days (Figure-2).

The number of cells per surface area (cells/mm^2) in 2D environment after 2 days of culture were on par for static (206 cells/mm^2) and 5 dynes/cm2 (239 cells/mm^2), whereas it significantly increased with shear stress of 10 dynes/cm^2 (327 cells/mm^2) and 15 dynes/cm2 (362 cell/mm^2), then significantly decrease for shear stress of 20 dynes/cm^2 (325 cell/mm^2). Following 4 days, the number of cells for shear stress of 15 dynes/cm^2 (636 cells/mm^2) was significantly ($p<0.05$) larger than those of static (385 cell/mm^2) and shear stress either of 5

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dynes/cm2 (456 cell/mm^2), 10 dynes/cm^2 (545 cells/mm^2) and 20 dynes/cm^2 (580 cells/mm^2) (Table-1).

For 3D environment culture studies, the number of cells per surface area (cells/mm^2) after 2 and 4 days of culture was significantly ($p<0.05$) larger for shear stress is 15 dynes/cm2 (345 and 587 cells/mm^2) than those static (221 and 412 cells/mm^2) and shear stress of 5 dynes/cm^2 (277 and 514 cells/mm^2), 10 dynes/cm^2 (345 and 587 cells/mm^2) and 20 dynes/cm^2 (369 and 645 cell/mm^2) (Table-1).

Table-1. Effect of dynamic shear stress on cells proliferation in 2D and 3D environment using flow culture chamber system.

Parameter	Culture time (days)	Number of shear stresses (dynes/cm^2)				
		0	5	10	15	20
Number of cells per surface area (cells/mm^2) in 2D environment	2	206^d±5.1	239^c±8.4	327^b±2.3	362^a±4.1	325^b±8.5
	4	385^c±2.4	456^c±4.1	545^b±5.1	636^a±6.3	580^b±1.2
Number of cells per surface area (cells/mm^2) in 3D environment	2	221^c±1.7	277^c±3.1	345^b±3.6	413^a±1.4	369^b±4.7
	4	412^e±1.0	514^d±1.1	587^c±9.1	700^a±4.1	645^b±1.8

Surface area is 400mm^2
Means in a row followed by different letters are significantly different

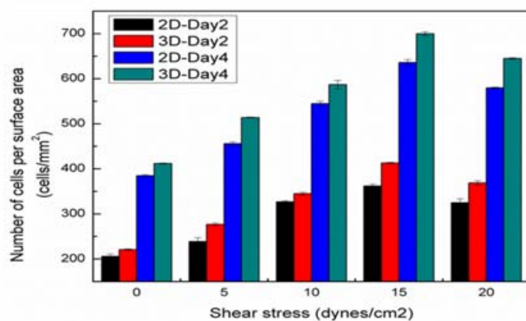

Figure-2. Number of cells on static and dynamic culture after 2 and 4 days culture studies. Flow culture chamber system in 2-dimensional and 3-dimensional.

Cell orientation, cell-to-cell connection and microvessel formation

The presented of shear stress also effect the morphologies of HUVEC as shown in Figure-4. HUVEC under shear stress 5-20dynes/cm^2 oriented and aligned parallel to the flow (flow was left to right) (Figure-3 (c-j)) compared to HUVEC under static which oriented and aligned randomly (Figure-3 (a & b)). After 2 days some HUVEC were more elongated for the culture system with 10-20dynes/cm^2 shear stress (Figure-3 (e, g & i)) than culture system under static and 5 dynes/cm^2 (Figure-3 (a & c)). The elongations of HUVEC were maximum under shear stress environment after 4 days and some HUVEC under static environment start to elongate.

The effect of shear stress over cell connections and microvessel development can be observed in Figure-4. HUVEC formed the microvessel in 3D environment (fibrin-HUVEC) and not in 2D environment (HUVEC only). In 2 days, HUVEC degraded the fibrin, connected each other and formed microvessel after 4 days. HUVEC under 15 dynes/cm2 have good growth and better connections after 2 days (Figure-3(g)) and fully formed microvessels after 4 days (Figure-3 (h)) compared with other culture environment. While under static and 5 dynes/cm^2, HUVEC needed more time to develop connections and lower density of HUVEC connections after 2 days (Figure-3(a & c)).

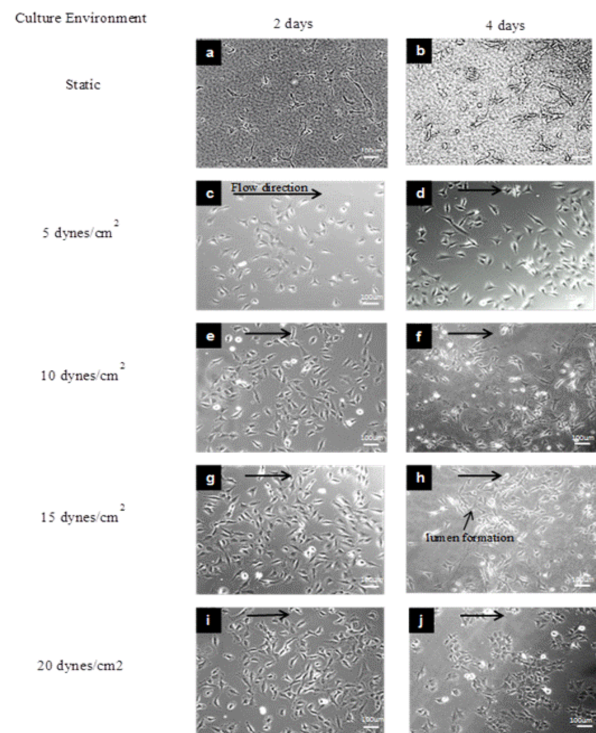

Figure-3. Cell orientation, elongation and microvessel development compared under static and flow condition in three-dimensional culture. HUVEC built cell connections, as observed by phase contrast microscopy, by day 2 (left-hand column) as well as by day 4 (right-hand column). Images of static culture environment (**a&b**), 5 dynes/cm^2 (**c&d**), 10 dynes/cm^2 (**e** &**f**) 15 dynes/cm^2 (**g** & **h**) and 20 dynes/cm^2 (**i&j**). The flow direction is left to right. Scale bars = 100μm.

HUVECs oriented, elongated (Figure-4 (b)) and then connected each other (Figure-4 (a)) to form microvessel. The formation of microvessel was verified by fluorescence microscopy for shear stress of 15 dynes/cm2 (Figure-4 (d)) showed the presence of lumen inside the network of cell connections. The phase contrast image of microvessels was taken as comparison (Figure-4 (c)).

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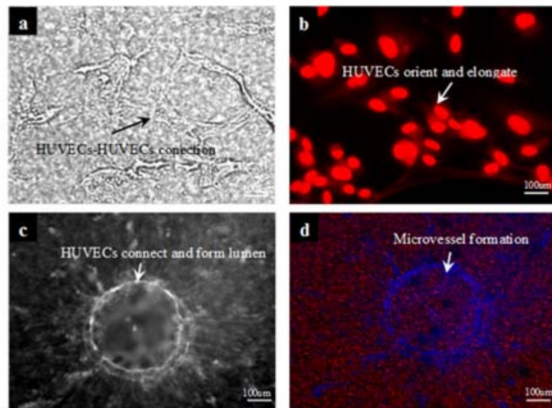

Figure-4. After 4 days of culture, HUVECs were oriented and elongated (b) and connected each other (a). HUVECs were stained with rhodamine-phalloidin for actin filament and Hoechst 33258 to highlight the position of the nuclei during imaging analyses. Pictures were recorded and present in (**d**) for higher magnification of the lumen (arrow) and (**c**) is a phase contras picture of microvessel. Scale bars = 100 µm.

DISCUSSION

The investigations of cells and tissues culture in vitro needs specific cultivation environments mimicked in vivo system. 3D environments and dynamic culture system are the accurate and precise of the needs of cells and tissue development and promise numerous advantages over 2D and static environment as also suggested by other researchers [18-19]. In vivo, cells are supported by a complex 3D extra-cellular matrix (ECM), which facilitates cell–cell communication and also provides mechanical support and chemical cues for cell adhesion, migration, proliferation, and differentiation [20]. In this study, we used fibrin gel as a 3D ECM to promote the ability of HUVEC successfully demonstrated in the present study. The fibrin gel has better matrices for cell adhesion because of: (1) it's very favorable wound-healing inductive capacities, (2) contains several binding motifs like RGD binding peptides that can stimulate cell responses [21], (3) porous meshing for better nutrient, gas as well as waste transport [22] and (4) provide free energy surface that favors cell adhesion.

The existence of shear stress is an important parameter that affects the stimulation of cell adhesion as well as microvessels formation. In this study, we demonstrated that shear stress induced by our flow culture chamber system, encouraged HUVEC proliferation and migration (Figure-3), thus enhancing the cell-cell connections (Figure-3) and promoted the 3D microvessels formation (Figure-4). Our recent studies have demonstrated that dynamic shear stress with a significant forward flow inhibited HUVEC proliferation and migration, when compared to static (Figure-2). HUVEC proliferation and migration rate have relationship with the rate of DNA synthesis, which higher DNA synthesis rate enhanced HUVEC proliferation and migration [23-24]. Dynamic shear stress has shown the increased rate of DNA synthesis, thus enhanced HUVEC proliferation and migration [25]. The optimal number of shear stress effect on HUVEC proliferation was also investigated and it is suggested that the appropriate number of shear stress for HUVEC function in vitro studies was 10-20dynes/cm^2, similar with in vivo [26-28].

Through this study, it was revealed that HUVEC were aligned, oriented and elongated due to dynamic flow (Figure-3). HUVEC were growing in random directions in static. Meanwhile, when cultured under shear stress, the HUVEC were more oriented and aligned to the flow direction. Numerous existing studies reported about the endothelial cell alignment that induced by shear stress and resulted from the dynamic flow [29-32]. The results also shown that the dynamic shear stress inhibited HUVEC to elongate more and facilitate the HUVEC to make cell-to-cell connection [33-34].

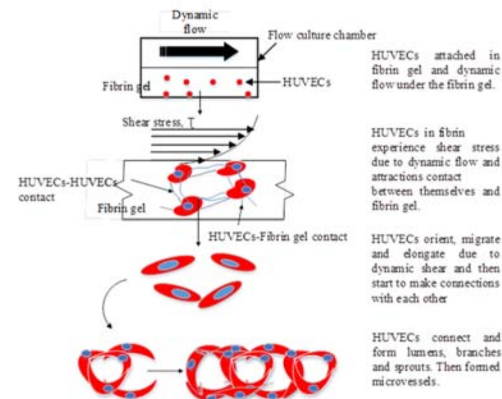

Figure-5. Schematic illustration of the effect of dynamic flow on microvascularization process.

In this study, the effect of dynamic shear stress in formation of functional and matured microvessel was also evaluated. The formation of microvessel only exists in 3D culture studies and not in 2D culture studies. Microvascularization and angiogenesis were referred to the growth of new microvessel (capillaries) by endothelial cells migrating and proliferating from the pre-existing blood vessel. Endothelial cells will orient and connect with neighboring cells to form a tubes network (tubes like structures) with the formation of lumens and initiate new blood vessels [35].

Through this study, we successfully reconstructed microvessel (Figure-4) that mimics the in vivo [36]. The application of shear stress in promoting the microvessel formation with induced HUVEC to degrade their surrounding extracellular matrix which facilitated HUVEC to proliferate, migrate and communicate each other's to form lumen formation within the endothelial sprout. A complete schematic illustration of the proposed microvascularization development due to dynamic shear stress is shown in Figure-5.

CONCLUSIONS

The effects of dynamic parameter (flow rate and shear stress) over 3D culture system in which human

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umbilical vein endothelial cells (HUVEC) were cultured in flow culture chamber inside fibrin gel has been characterized and justified in this study. Dynamic shear stresses were shown to enhanced HUVECs proliferation and migration, thus increasing the number of cell-cell connections and consequent microvessels development compared to static environment. There were less cell-cell connections presented in static environment and no formation of microvessels after 4 days culture period. The existence of shear stress due to dynamic flow was also promoted more mature microvessels containing more cell-cell connections and contains lumens especially. This culture process concludes that dynamic shear stress was an important factor influence in vitro and in vivo cell behavior during the growth and the regeneration of cellular tissue.

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