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IN VITRO MODELS FOR ANGIOGENESIS

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ABSTRACT

Angiogenesis, the formation of new blood vessels, is an essential process for tissue and organ development, wound healing and pathological events. To understand the mechanism of angiogenesis, choosing a suitable model for research is important and sometimes difficult. This review introduces some of the current and popular *in vitro* models for angiogenesis research, especially for investigating the morphological differentiation process of endothelial cells, including two-dimensional and three-dimensional models. The fabrication methods, advantages, limitations of each model and some future perspectives are discussed.

Keywords: Angiogenesis, cell sheet, endothelial cells.

Các mô hình *in vitro* cho nghiên cứu quá trình tạo mạch máu

TÓM TẮT

Quá trình sinh mạch, sự tạo thành mạch máu mới từ những mạch máu cũ, là một quá trình thiết yếu cho sự phát triển của các mô, cơ quan, quá trình làm lành vết thương, cũng như các quá trình gây bệnh. Để nghiên cứu cơ chế của quá trình sinh mạch, việc lựa chọn mô hình nghiên cứusao cho phù hợp rất quan trọng và đôi khi không dễ dàng. Bài tổng quan này giới thiệu một số mô hình *in vitro* phổ biến cho nghiên cứu quá trình sinh mạch, đặc biệt chú trọng nghiên cứu quá trình biệt hóa hình thái của tế bào nội mô, bao gồm các mô hình hai chiều và ba chiều. Phương pháp chế tạo, ưu nhược điểm của từng mô hình, cũng như sự phát triển của quá trình sinh mạch trong tương lai sẽ được thảo luận.

Từ khóa: Quá trình tạo mạch, tấm tế bào, tế bào nội mô.

1. INTRODUCTION

Tissue engineering is an interdisciplinary field that grounds on the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (Langer and Vacanti, 1993). The original goal of tissue engineering was to fabricate new and functional tissues using living cells associated with matrix or scaffolding in order to solve the critical gap between the growing number of patients on the waiting list for organ transplantation due to end-stage failure and the limited number of donated organs available for such procedures (Lavik and Langer, 2004). Many significant progresses have been seen in tissue engineering including many approaches on material designs, reactor designs or cell sources (Lavik and Langer, 2004). However, beside some solved problems and developments, it is necessary to mention about the greatest and unsolved challenge in tissue engineering which is well-known as angiogenesis (Griffith and Naughton, 2002; Lavik and Langer, 2004).

Angiogenesis, the process of new capillary formation from the pre-existing vessel, has been attracting many interests since it plays a very important role in the development of embryonic, reproductive function, wound healing in adults, as well as in many diseases, particularly cancer (Carmeliet, 2005; Folkman, 1995; Munoz-Chapuli et al., 2004; Simons, 2005). Depending on the balance of pro- and anti-angiogenesis molecules, endothelial cells (ECs) are quiescent, existing as a protective monolayer barrier within the blood vessels, or are rapidly activated to enter angiogenesis. During this process, ECs could migrate, proliferate, form and stabilize lumen structures (Bayless and Johnson, 2011; Jain et al., 1997). Successful control of angiogensis is expected to be the key to cure many types of diseases such as cancer, the ocular diseases, obesity, diabetes, cardiovascular disease (Cao, 2010), and improve the thickness of artificial tissue.

To understand the angiogenesis process, *in vitro* angiogenesis assays are very important. *In vitro* assay can be quickly and easily carried out using basic lab equipment with the flexibility of cell types, staining, observation methods, and quantitative analysis. However, it is sometimes difficult to choose the suitable assay since there are many points to be considered, e.g., the choice ofEC cell type, seeding pattern, the positions of ECs in the assay, mono or co-culture, culture medium, additional growth factors, static or dynamic culture, which are all dependent on experimental design. Last but not least, the use of either a two- or three-dimensional (2D or 3D) assay is also important. However, due to the rapid development of

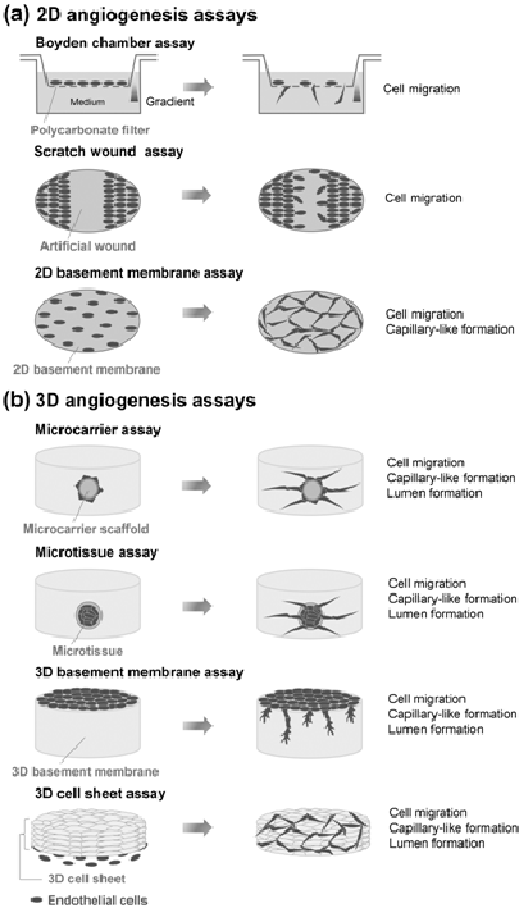

Figure 1. Current and popular *in vitro* angiogenesis assays (Ngo et al., 2014)

fabrication techniques in tissue engineering, it is sometimes difficult to choose a suitable one (Ngo et al., 2014).

In this paper, several popular 2D and 3D *in vitro* models, especially for investigating the morphological differentiation process of endothelial cells (Figure 1) and future perspectives of angiogenesis research are reviewed and discussed.

2. 2D ANGIOGENESIS MODELS

2.1. Boyden chamber assay

The Boyden chamber consists of two chambers divided by a polycarbonate filter with a pore size of 5–12 μm (Bahramsoltani et al., 2009) to test the chemotaxis migration of ECs. Using different extracellular matrix (ECM) protein, including gelatin, collagen, fibronectin, or complex matrices such as Matrigel, the filter can be coated to improve the cell attachment or to test the cell-substrate interaction (Albini and Benelli, 2007; Albini et al., 2004). ECs are seeded on the upper chamber and culture medium containing pro- or anti-angiogenic factors is loaded into the lower chamber to observe the chemotaxis migration of ECs. Gradient of angiogenic factors could also be prepared by a cancer cell layer seeded under the filter as a non-contact assay (van Moorst and Dass, 2011). Using time-lapse observation and image processing, the migration of Ecs through the filter is investigated easily (Iwahana et al., 1996). Boyden chamber allows experiments with high sensitivity, high reproducibility and short duration (4–6h)(Staton et al., 2009). However, it is difficult to observe the vertical migration through a 'foreign' matrix (polycarbonate or polypropylene filter)(Staton et al., 2009).

2.2. Scratch wound assay

The scratch wound assay is a simple method to observe and measure the horizontal migration of ECs and other cell types. Briefly, an artificial wound in a certain area on a confluent monolayer of ECs is created by mechanically scratching using a cell scraping tool (Wong and Gotlieb, 1984) or using a laminar flow of trypsin solution (Nie et al., 2007). The wound can also be prepared by placing a Teflon ring in the middle of the culture dish. After cells becomes confluence, the ring is removed to create an empty space without cells as an artificial wound. Then, the migration of Ecs into the "wound area" is observed using time-lapse microscope and quantitatively analyzed using image processing (Bahramsoltani et al., 2009; Glen et al., 2012; Pepper et al., 1990; Wang et al., 2009; Weis et al., 2002). This assay allows the quick (several hours to overnight) and easy monitoring of horizontal cell migration, but might be not accurate since cell migration here also involves cell spreading and proliferation (Coomber and Gotlieb, 1990; Staton et al., 2009).

2.3. 2D basement membrane assay

The 2D basement membrane assay is one of the most popular *in vitro* assays for cell migration and capillary-like formation during angiogenesis. In this assay, ECs are seeded on a surface coated with collagen, laminin, fibrin and Matrigel, or are covered by a thin layer ofthese basement membranes (Arnaoutova et al., 2009). The optimal amount of these matrix should be considered during assay set up, since they may affects EC behaviors (Staton et al., 2009). Subsequently, using conventional microscopy, cell attachment, migration and capillary-like formation are easily observed and analyzed over 4 to 24 hrs. This assay has been used to evaluate the effect of many pro- and anti-angiogenic factors, as it is reliable, quick, quantitative, sensitive, adaptable to high-throughput, easy to perform (Arnaoutova et al., 2009). Nevertheless, tubule formation of some non-endothelial cells, such as fibroblasts or cancer cells, is observed on Matrigel, indicating that this model might not specific for ECs (Auerbach et al., 2003). In addition, the capillary-like formation does not require cell migration and proliferation on Matrigel (Kubota et al., 1988). Hence, the problem of this assay is

that it lacks 3D structure and cannot imitate some mechanical and biochemical signals present *in vivo*.

3. DANGIOGENESIS MODELS

3.1. Microcarrier assay

Using this method, microcarrier scaffolds, made of plastic, glass, dextran, cellulose, collagen (Malda and Frondoza, 2006) and fibrin (Nakatsu and Hughes, 2008) with a suitable diameter (typically 100-400 mm), are coated with gelatin to enhance the attachment of ECs on their surface (Nehls and Drenckhahn, 1995). After EC attachment (2-4 days), microcarriers are embedded in a fibrin matrix. Then, the migration and capillary-like formation of ECs are observed and quantitatively analyzed. The ECM type and density used for embedding the microcarriers strongly affects endothelial cell behavior (Ghajar et al., 2008; Kniazeva and Putnam, 2009; Nehls and Drenckhahn, 1995). This model can avoid the EC detachment as in the 2D models, has better mimics the *in vivo* environment, and enhances endothelial tube formation. The flexibility of microcarrier coatings is an excellent tool to study cell-cell and cell-surface interactions for angiogenesis (Hall and Hubbell, 2004; Kim, 2005). In spite of this, the observation of cell migration is limited mainly in a 2D horizontal *X*-*Y* plane, likewise in the case of 2D models.

3.2. Microtissue assay

Microtissue is a scaffold-free spherical aggregate of cells and can be fabricated using different techniques, including pellet culture, spinner-flask culture, hanging drop culture, liquid overlay, rotating wall vessel culture(Achilli et al., 2012). The formation of spherical aggregatesbases on the prevention of cellattachment to the culture surface (Kelm and Fussenegger, 2010). Microtissuesize shouldbe optimized (100-500 mm of diameter) by changing the cell number. Otherwise, it will lead to the limitation of diffusion, accumulation of metabolic waste and hypoxia inside the microtissue core, leading to the necrotic core formation (Kelm and Fussenegger, 2010).

Vernon et al. introduced a model, termed“radical invasion of matrix by aggregated cells” (RIMAC) for EC migration and morphogenesis (Vernon and Sage, 1999). A single microtissuewith adefined number of ECs is prepared using the “hanging drop” method, then coated with hardened collagen type I and incubated with medium containing growth factors to be tested for 2-5 days. Subsequently, migration of ECs into the collagen is observed and analyzed in the horizontal *X*-*Y* plane. This *in vitro* model does not fully simulate vascular growth *in vivo*, but its simplicity and reproducibility should make it useful for screening pro- and anti-angiogenic factors.

Microtissue, which is a living assembly of cells via self-assembly in a scaffold-free environment, provides more biological information, requires low cell number, is inexpensive, easy to prepare, permits flexible cell-type composition,andis applicable to high-throughput drug screening (Friedrich et al., 2009; Kunz-Schughart et al., 2004). However, observation and analysis of cell behavior in the microtissue is oftendifficult because ofits spherical shape and thickness. Sectioning using a microtomeor application of several advanced imaging technologies (e.g., multi-photon confocal laser scanning microscopy) is required for larger microtissue. In addition, it is also difficult to control the initial position of Ecs in the heterogeneous microtissue.

3.3. 3Dbasement membrane assay

The 3D basement membrane assay can be prepared easily from 2D basement membrane assay described above by increasing the matrix thickness (Deroanne et al., 1996). Several seeding patterns (single cells or monolayer) and seeding positions (bottom, middle or top of the 3D scaffold) of ECs can be controlled flexibly. ECs actively migrate, elongate, connect and organize into a network of tube-like structure which then regress if the 3D basement membrane is removed (Deroanne et al., 1996).

The 3D basement membrane assay is becoming one of the most frequently used models for quantitation of angiogenesis *in vitro,* since it has many advantages compared tothe 2D assay (Bahramsoltani et al., 2009). It supports ECs in formingnot only capillary-like structures, but also lumens. The EC behaviorscanbe observed and quantitatively analyzed both in horizontal and vertical aspects(Bayless and Davis, 2003). However, this assay take longer time to run (5-15 days) (Staton et al., 2009) and the 3D matrix could be degraded due to the secreted proteins during cell migration. In addition, the thickness of the 3D matrix must be optimized to avoid the cell necrosis.

3.4. Cell sheet assay

The 3D scaffold-free cell sheet is becoming a convenient and popular model for angiogenesis. Briefly, cells are cultured to confluence as a monolayer, harvested without using proteolytic enzymes to preserve the sheet structure, and stacked to form a multilayered sheet (Haraguchi et al., 2012a; Ngo et al., 2013; Nishiguchi et al., 2011). Cell sheets could be prepared by using other different techniques, including a thermo-responsive polymer-grafted surface(Okano et al., 1993), a biodegradable peptide-grafted surface (Qiu et al., 2010),a collagenase degradable atelocollagen film(Nagai et al., 2004), a magnetic-force based tissue engineering technique (Ito et al., 2005), a layer-by-layerassembly technique with ECM-coated cells (Matsusaki et al., 2007), and a bioprinting method (Nakamura et al., 2010).

Among mentioned techniques, thermo-responsive polymer-grafted surface is the most widely-used technique. Herein, a layer of *N*-isopropylacrylamide (IPAAm) monomer is applied on a normal culture surface and then subjected to electron beam irradiation, leading to the covalent immobilization of the polymer (PIPAAm) onto the surface (Okano et al., 1993). A smart characteristic of the PIPAAm-grafted layer is that it is temperature-responsive (Haraguchi et al., 2012b). Above 32°C, the surface is hydrophobic, allowing cells to attach and proliferate to a confluent monolayer. Below 32°C, the surface is hydrophilic and the monolayer cell sheet can be easily detached from the surface without using enzymatic treatment. By using this method, the cell-cell connections and accumulated ECM are preserved for maintaining better biological cell function. This method works well with many cell types and support the flexibility of different EC seeding patterns (Kino-oka et al., 2012; Nagamori et al., 2013; Ngo et al., 2013;Sekiya et al., 2006; Shimizu et al., 2003; Asakawa et al., 2010;Kanayama et al., 2007; Miyahara et al., 2006; Ohno et al., 2009).

Our group have developed a 3D *in vitro* model, including a multilayered myoblast sheet overlaying ontohuman umbilical vein endothelial cells (HUVECs), to mimic *in vivo* angiogenesis after transplantation of a myoblast sheet for treating myocardiac infarction (Nagamori et al., 2013). With live staining, cell behavior could be easily observed using a time-lapse confocal laser scanning microscope. Cell spatial localization in the 3D cell sheet and extent of HUVEC network formation could be quantitatively analyzed using image processing. We observed that myoblast cells actively migrated inside the cell sheet, making sheet fluidity (Kino-oka et al., 2012). HUVECs were found to elongate, actively migrate using their expanded filopodia and connect to form network with lumen structure after 4 days of incubation (Nagamori et al., 2013; Ngo et al., 2013).The cell sheet thickness and HUVEC seeding densitywerefound to have strong influence on HUVEC behaviors in the 3D cell sheet.

The 3D model using cell sheets allows the observation and analysis independently in 2D horizontal (*X*-*Y* plane) or 1D vertical (*Z* axis) directionusing a confocal laser scanning microscopy and image processing. The initial position of the ECs can also be easily controlled, enhancing flexibility in research design. The cell number, size and thickness of cell sheet arecontrollable to apply in high-throughputassays. Nevertheless, this method

requiresa hugenumber of cells to enable its fabrication and is difficult to handle because of cell sheet fragility.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In this review, some of the current and popular *in vitro* angiogenesis models are introduced. So far, the ideal *in vitro* angiogenesis model, which should resemble the angiogenesis process being studied, should be easily andrapidly performed with reliable results and using automated computational analysis, should permit multi-parameter assessment, and should relate directly to the results seen in the clinic,has not been developed yet (Staton et al., 2009). Hence, the combination of severalabove assays may be essential to enable better interpretationof the results.

It is clearly seen that 3D *in vitro* models have many advantages and better reflect the native organs and tissues *in vivo*, compared to 2D models. 3D models support ECs to fully express their natural behavior during the angiogenesis process, for example permitting cell migration, proliferation, connection and lumen formation. Following the discussion herein, 3D *in vitro* cell sheet models, which facilitate full observation and quantitative analysis by simplifying a 3D arrangement into 2D (*X*-*Y* plane) and 1D (*Z* axis), might be more widely used into the future.

In the future, researchers should focus on the improvement of thicker 3D structure with enhanced capillary and lumen formation. Moreover, the sample size should be small enough to save the required cell number and applicable for high-throughput screening. Along with the thickness of sample, it is necessary to improve the microfluidic flow of medium to the sample to ensure sufficient supply of nutrients and oxygen for cell survival, as well as to study the effect of shear stress (Hsu et al., 2013; Inamdar and Borenstein, 2011). In addition, advanced imaging techniques should be developed to enable the observation of thicker tissue with automatic image processing.

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