Chapter 9

Miniaturized Assays of Angiogenesis In Vitro

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Abstract

Assays of angiogenesis in vitro provide insights into vascular development and are useful in studies of agents that modulate blood vessel formation. This chapter describes techniques to induce angiogenesis-like sprouting from aortic and microvascular explants cultured in 3-dimensional native collagen gels. The chapter focuses on explants derived from mice and use of a miniaturized format that permits efficient utilization of reagents, ease of processing, rapid analysis, and conventional imaging.

Key words: Mice, Fat, Microvessels, Aorta, Rings, Angiogenesis, 3D, Collagen

1. Introduction

Angiogenesis, which is the generation of new vasculature from preexisting blood vessels, is a critical component of normal reparative processes such as wound healing and pathological processes that include diabetes, scleroderma, and cancer. Models of angiogenesis in vitro have proven useful in studies of vascular morphogenesis and age-related changes in neovascularization, and also as assays to screen compounds for therapeutic regulation of blood vessel growth. The generation of blood vessel (capillary)-like structures in vitro occurs best in 3-dimensional (3D) extracellular matrix (ECM) gels comprised of collagen, fibrin, or basement membrane material that simulate the connective tissue environment in vivo. Typical models utilize either isolated, cultured endothelial cells (ECs) or vascular explants as starting material. The latter are comprised of segments of macro- or microvessels that contain an endothelium and associated supportive (mural) cells.

3D ECM-based models utilizing isolated ECs can generate credible vascular structures. For example, human umbilical vein

ECs (HUVECs) dispersed in type I collagen gels can be reliably induced to develop tubes with wide, patent lumens that closely resemble capillaries in vivo (1, 2). Like HUVECs, adult human microvascular ECs, under specific conditions of stimulus, can also develop into tubular structures with patent lumens (3). Models that incorporate isolated ECs are used widely, but have been criticized on the basis that their genesis of capillary-like structures is more representative of early embryonic vasculogenesis rather than true angiogenesis, which requires a “parent” blood vessel as the source of new sprouts. This potential shortcoming has been addressed by the development of 3D ECM-based models that induce sprout formation from explanted vascular segments. Substantial success has been achieved using explants of adipose tissue-derived microvessels and aortae from rats (4–11). However, the multiplicity of powerful genetic tools developed for mice has provided a strong incentive to devise models of angiogenesis that use aortae and microvasculature explanted from mice (12–15).

Vascular explants, by virtue of their shape and volume, require a 3D ECM gel in which to sprout. A form of ECM used frequently for in vitro culture systems is native type I collagen extracted from bovine dermis or the tail tendons of rats (16–18). In addition to these sources of type I collagen (which are available commercially and are relatively affordable), a growing interest in studying angiogenesis in mice has led to the use of 3D gels of mouse type I collagen as supports for mouse ECs or vascular explants. Although commercial sources of murine collagen exist, it is currently more cost-effective for the investigator to prepare their own extracts. Therefore, methods for extraction of native type I collagen from mouse tails are outlined in Subheading 3.3.

In addition to considerations regarding the sources of cellular starting material and ECM, there exists the ever-present problem of scarcity and/or expense of bioactive compounds selected for study, which has created the need for angiogenesis assays in miniaturized formats. To address this issue, an EC-based angiogenesis assay was developed in 1999 that utilized rings of nylon mesh to support lenticular collagen gels that were small enough to fit in a standard 96-well assay plate (19). This miniaturized approach was subsequently adapted for use with murine microvascular explants and aortic explants (14), which are the focus of this chapter.

2. Materials

2.1. Murine Microvascular Explants

1. Epididymal fat pads from 3 to 5 mice (6–10 fat pads).
2. 60-mm diameter cell culture dishes.
3. Small dissecting scissors.

4. Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin).
5. Bovine serum albumin.
6. Type II collagenase.
7. Fetal bovine serum.
8. 50-mL Erlenmeyer flask.
9. Disposable plastic syringes (one 10 mL and two 20 mL).
10. Two Swinnex® filter holders (25 mm diameter) (Millipore Corp., Billerica, MA).
11. 30-µm mesh nylon screen (Sefar America, Inc.).
12. 300-µm mesh nylon screen (Millipore Corp.).
13. 18-gauge needles.
14. Centrifuge, rotary incubator/shaker.

2.2. Murine Aortic Explants

1. Mouse aortae, removed surgically.
2. 60-mm diameter cell culture dishes.
3. MCDB 131 medium (Gibco/Invitrogen) supplemented with antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin).
4. #20 scalpel blade on a size #4 handle.
5. Coarse-tipped (#1) and fine-tipped (#3, or #5) watchmaker's tweezers.
6. Small dissecting scissors.

2.3. Extraction of Mouse Tail Collagen

1. Isolated, skinned mouse tails (3–5 per preparation).
2. Strong tweezers or hemostat.
3. #10 scalpel blade on a size #3 handle.
4. Small dissecting scissors.
5. 50 mM Tris-buffered saline, pH 7.4–7.6 (TBS).
6. Acetone.
7. 70% isopropanol.
8. 0.05 N acetic acid.
9. Sircol™ Soluble Collagen Assay (Accurate Chemical and Scientific Corp., Westbury, NY).

2.4. Preparation of Collagen Solution for 3D Gels

1. Collagen extract (Subheading 3.3).
2. 10-strength Medium 199 (Gibco/Invitrogen).
3. Dulbecco's Modified Eagle Medium.
4. Fetal bovine serum.
5. 0.1 N HCl or NaOH (for adjustment of pH).

2.5. Fabrication of 3D Collagen Gel Assemblies and Culture of Explants

1. Cold-stamped rings made from nylon (Nitex) mesh (Sefar type 03-100/44) with inner and outer diameters of 3 and 5.6 mm, respectively (Sefar America, Inc.).
2. 60-mm diameter plastic cell culture dishes.
3. Parafilm M™ (for lining the plastic culture dishes) (Pechiney Plastic Packaging, Chicago, IL).
4. Human vascular endothelial growth factor 165 (VEGF₁₆₅) (PeproTech, Rocky Hill, NJ) 10 ng/mL in phosphate-buffered saline (PBS).
5. Filter paper.
6. Mouse tail collagen solution (Subheading 3.4).
7. 96-well cell culture plates.
8. MCDB 131 medium with antibiotics.
9. Fetal bovine serum.

3. Methods

3.1. Murine Microvascular Explants

Typically, murine microvasculature for explants is obtained from masses of highly vascularized fat, such as that surrounding the epididymis (4, 5, 15, 20). Sprout development from these explants is considered to be representative of angiogenesis insofar as there are parent microvessels comprised of ECs and mural cells from which the sprouts emanate. The model is characterized by spontaneous growth in defined media with minimal serum, clear evidence of lumen formation, and an easily quantified sprouting response.

1. To isolate microvessels, epididymal fat pads from 3 to 5 male mice are pooled, gently cut with small dissecting scissors into cubes 1–2 mm on each side, and placed in 20 mL of DMEM containing 2 mg/mL of fatty acid free, low endotoxin, cell culture tested bovine serum albumin, and 2 mg/mL of Type II collagenase prewarmed to 37°C.
2. The suspended fat is placed in a 50-mL Erlenmeyer flask and agitated at 37°C in a rotary incubator/shaker at 120–160 rpm until the fat is digested into a slurry (digestion requires approximately 20–30 min).
3. Blood vessels are separated from adipocytes by centrifuging the mixture at 1,000×g for 5 min (the adipocytes float to the top while the vasculature is pelleted).
4. The vascular pellet is washed by gentle resuspension in 8 mL of DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics followed by 3 min of centrifugation at 1,000×g. Washing is performed twice.

5. The pellet is then resuspended in 8 mL of DMEM/10% FBS/antibiotics and poured through a 20-mL syringe body fitted with a 25-mm diameter Swinnex filter holder containing a 300- μ m mesh nylon screen in order to remove tissue fragments and pieces of large-caliber vasculature.
6. The filtrate, consisting primarily of microvascular segments and single cells, is then passed through a 20-mL syringe body fitted with a 25-mm diameter Swinnex holder containing a 30- μ m mesh nylon screen which traps the microvascular segments, but allows single cells to pass through.
7. Subsequently, the microvascular segments are washed from the 30- μ m mesh screen into a 60-mm diameter cell culture dish by jets of DMEM/10% FBS/antibiotics delivered from a 10-mL syringe fitted with an 18-gauge needle.
8. Typical isolates consist of 20–40 microvascular segments with lengths of 30–150 μ m. The isolated segments are suspended in 3D collagen gels within 30 min, as described in Subheading 3.5 (see Note 1).

3.2. Murine Aortic Explants

Previous studies have shown that explanted segments of rat aortae placed in 3D ECM gels will give rise to arborizing, capillary-like sprouts with an endothelial lining (6–11). Coincident with the use of microvascular explants derived from murine adipose tissue, an ongoing interest in studying genetically modified mice has led to the use of mouse aortic segments as an alternative to rat segments. Under permissive conditions in vitro, mouse aortic segments will generate microvascular sprouts similar to sprouts formed by rat segments (12–14).

1. To obtain aortic segments from mice, the aorta is identified by its location with respect to the heart. The aorta is more easily obtained from the posterior wall of the thoracic cavity after careful removal of the lungs.
2. The aorta is cut at the arch and at its junction with the diaphragm and transferred to a sterile 60-mm diameter cell culture dish containing 5 mL of MCDB 131 medium supplemented with antibiotics.
3. Isolated aortae are rinsed twice with this medium to remove extravasated blood and then are gently cleaned of loose perivascular connective tissue. A complete removal of this connective tissue minimizes fibroblast contamination of the explant.
4. The cleaned aortae are cut transversely with a scalpel (fitted with a #20 blade) into segments 1 mm in length. The cut is made by moving the curved edge of the scalpel blade through the tissue with a single rocking motion, which minimizes damage to the endothelial lining.

5. Subsequently, the aortic segments are rinsed in the MCDB 131 medium and, within 30 min, are placed into 3D collagen gels, as described in Subheading 3.5 (see Note 2).

3.3. Extraction of Collagen from Mouse Tails

Although a variety of ECM hydrogels (type I collagen, fibrin, basement membrane matrix) support vascular sprout formation in vitro, we and others have found that type I collagen extracted from mouse tail tendons is an effective, relatively inexpensive support. Notably, mouse tail collagen permits experimentation in an “all-mouse” system in which the ECM support and vascular explant can be matched syngeneically (21).

1. To prepare collagen extracts, tendons are peeled from the skinned tails of 3–5 mice by pulling the tendons away from the vertebral bodies with a strong tweezers or hemostat while cutting the tendons’ connections to the vertebrae with #10 scalpel blade fitted to a #3 handle. More than 3 mice are needed if the animals are greater than 18 months of age.
2. The isolated tendons are pooled, minced with small dissecting scissors, and immersed for sequential, 5-min periods in 50 mM TBS, acetone, and 70% isopropanol.
3. Type I collagen is extracted by stirring the tendons overnight in 50 mL of 0.05 N acetic acid at 4°C.
4. The resulting viscous solution is centrifuged at $4,000\times g$ for 15 min at 4°C to remove insoluble material.
5. Collagen content (which is typically between 1.5 and 2.5 mg/mL) is quantified by the Sircol™ Soluble Collagen Assay. The Sircol assay is specific for collagen and does not detect proteoglycans, elastin, or other ECM components.
6. Collagen extracts should be stored under refrigeration at 4°C, but never frozen.

3.4. Preparation of Collagen Solution for 3D Gels

1. 3D collagen gels are prepared by combining 1 volume of collagen extract (Subheading 3.3), 1/9 volume of 10-strength Medium 199, and sufficient DMEM and FBS to yield a solution with final collagen and FBS concentrations of 1.5 mg/mL and 2.5%, respectively. If necessary, the pH of the collagen solution is adjusted to 7.0–7.2 with 0.1 N HCl or NaOH.
2. The collagen solution is kept on ice prior to dispensing it over ring-shaped supports of nylon mesh, as described in Subheading 3.5.
3. To provide additional buffering capacity, the 10-strength Medium 199 can be supplemented with powdered NaHCO_3 , added to saturation.

3.5. Fabrication of 3D Gel Assemblies and Culture of Explants

Although there is consensus that 3D ECM gels are optimal for support of angiogenesis in vitro, the fragility of these gels can make analyses (i.e., staining, imaging) problematic. Moreover, gels of

relatively large size, which are typically easier to handle and image, are wasteful of scarce and expensive reagents. We have found that explants of murine microvasculature and aortae can be embedded within low-volume (30 μ L) 3D collagen gels supported at the edges by rings of nylon mesh. This approach combines the extensive capabilities of the mouse experimental system with a culture method that optimizes specimen handling, staining, and economical use of reagents. Of note, the clear lenticular gels can be imaged by conventional, wide-field microscopy in addition to confocal microscopy.

Assemblies comprised of nylon mesh rings, 3D collagen gels, and vascular explants are prepared as follows:

1. Custom-made, cold-stamped nylon (Nitetex) mesh rings (mesh type 03-100/44) with inner and outer diameters of 3 and 5.6 mm, respectively, are placed on 60-mm diameter plastic cell culture dishes lined with UV-sterilized sheets of Parafilm MTM to create a hydrophobic surface.
2. The rings are then flooded with 10 μ L of the mouse tail collagen solution described in Subheading 3.4 and the dishes covered with their plastic tops, which are lined with moist filter paper.
3. After 30 min in a tissue culture incubator at 37°C/5%CO₂/100% humidity to gel the collagen, 3–5 microvessels (suspended in a small volume of culture medium) or single aortic segments are placed on the collagen gel, carefully overlaid with an additional 20 μ L of the collagen solution, and returned to the incubator for 30 min to gel the collagen overlay.
4. The assemblies are then transferred to 96-well cell culture plates filled with 50 μ L (per well) of MCDB 131 medium supplemented with 0–2.5% FBS, and antibiotics. VEGF₁₆₅ added at 10 ng/mL serves as a positive control for induction of vascular growth.
5. After 3–10 days of culture (with media changed every 2 days), the assemblies are rinsed in PBS, fixed 20 min in 10% neutral-buffered formalin (NBF), and stored in PBS prior to analysis (Subheading 3.6).

3.6. Analyses of Sprouts Produced by Microvascular and Aortic Explants

1. Embedded microvascular explants (Fig. 1) give rise to multicellular sprouts after 2–4 days. Emergent single cells are excluded from analysis.
2. Typically, multicellular sprouts grow from embedded aortic segments after 4 days (14) (Fig. 2).
3. After fixation in NBF, the nuclei of sprouts can be labeled with conventional nucleic acid dyes such as DAPI, propidium iodide, or TO-PRO[®]-3 iodide and their cytoplasm labeled with fluorescent (e.g., Alexa-fluor[®])-conjugated phalloidin, which binds to f-actin.

Fig. 1. Murine microvascular segments embedded in a type I collagen gel. This preparation includes segments representing the largest (*black arrow*) and smallest (*white arrow*) examples within the typical range of segment sizes. Scale bar = 50 μm .

Fig. 2. A murine aortic segment cultured in a type I collagen gel for 7 days has produced a large number of sprouts (e.g., *arrow*). A field of single cells that have migrated from the segment is indicated by the *asterisk*. The supportive ring of nylon mesh can be seen at the periphery of the image. Scale bar = 200 μm .

4. After labeling, the 3D gel assemblies are mounted on slides and coverslipped using a photo-bleach resistive mountant, such as Vecta-Shield®.
5. The nylon mesh rings of the assemblies act as spacers that allow the collagen gels to be flattened slightly between the slide and coverslip, but not crushed. Consequently, the assemblies remain intact and can be demounted from the slides (several times, if necessary for washing and restaining) by immersing the slides on edge in PBS until the coverslips slide off.
6. As an alternative to imaging by fluorescence, fixed sprout preparations can be visualized by staining with a general protein stain,

such as 2% crystal violet for 10 min followed by multiple washes in PBS or TBS until the collagen gels became transparent.

7. Sprout formation from labeled preparations can be quantified by use of MetaMorph® (Molecular Devices, Sunnyvale, CA) or other image analysis software according to parameters of cell number, lumen diameter, and the number and length of primary, secondary, tertiary, and quaternary branches.
8. Measurements can be made using wide-field microscopy; however, greater discrimination, particularly with respect to luminal morphology, can be achieved by measurement of z-stacked images taken with a confocal microscope.
9. ECs can be distinguished from mural cells by uptake of DiI-acetylated low-density lipoprotein or by immunofluorescence with anti-CD31/PECAM antibodies.
10. Mural cells can be identified by their negativity for endothelial markers or by positive immunofluorescence for alpha smooth muscle actin.

3.7. Summary

In vitro models of angiogenesis within miniaturized, 3D ECM supports permit efficient visualization and measurement of vascular growth. Explanted microvessels or aortic segments obtained from mice of different ages or genetic backgrounds can act as parent blood vessels for angiogenesis-like sprout formation. 3D angiogenic models can be used to evaluate a multiplicity of factors that could regulate sprouting, including: (1) specific gene products expressed by ECs and/or mural cells, (2) the composition and physical characteristics of the surrounding ECM, and (3) exogenously added drugs, biological agents, or other chemical compounds with the potential to promote or inhibit angiogenesis.

4. Notes

1. *Murine microvascular explants*: Successful isolation of microvascular explants (Subheading 3.1) involves a number of factors, which include the initial size range of the cubed epididymal fat, specific activity of the Type II collagenase, and the time, temperature, and degree of agitation during digestion. Microvessels will not be released from under-digested fat, whereas over-digestion will result in fragile microvessels that will disintegrate during the washing and filtration steps. Even if isolation is successful, over-digested microvessels may die in culture. Establishing the optimal conditions for digestion that result in collection of viable microvessels requires a certain amount of trial and error.

2. *Murine aortic explants*: As mentioned in Subheading 3.2, the freshly isolated aortae should be gently cleaned of loose perivascular connective tissue prior to being cut into short segments for embedment in collagen. Removal of connective tissue can be accomplished by a combination of two techniques, which involve (1) holding the aorta at one end with a relatively coarse-tipped (e.g., a #1) watchmaker's tweezer and pulling the tags of connective tissue away with a fine-tipped (e.g., #3 or #5) tweezer held in the opposite hand, and (2) holding the aorta at one end with the coarse-tipped tweezer and using a small dissecting scissors to trim the connective tissue away. Scissors with the blades set at an angle to the handle are particularly useful, as the angle allows the blades to be brought parallel to the aorta for a close cut that removes superficial tissue without penetrating the aortic wall. After the aorta is cleaned of connective tissue, the portion of the aorta held by the coarse tweezer (which is usually crushed) can be trimmed away and discarded.
3. *Preparation of collagen solution for 3D gels*: Collagen extracts of mouse tail tendons (Subheading 3.3) and similar, commercial extracts of rat tail tendons or bovine dermis are comprised primarily of native collagen monomers. Adjustment of these extracts to physiological ionic strength and pH, followed by heating to 37°C (Subheadings 3.4, steps 1 and 3.5, step 3), causes the monomers to assemble spontaneously into long, banded fibrils 70–100 nm in diameter. Collectively, these fibrils form a meshwork with the physical properties of a malleable but dimensionally stable hydrogel. To avoid premature polymerization, it is important to chill all of the components of the collagen solution on ice prior to combining them and to keep the resultant mixture on ice prior to dispensing it onto the nylon mesh supports. Best results are obtained when the DMEM, 10-strength Medium 199, and FBS are combined first and the acidic collagen extract added last. All components should be combined in a centrifuge tube and mixed slowly by hand by rocking the tube end-over-end 5–8 times. Vortexers or magnetic stir bar-type mixers should be avoided, as energetic mixing can cause the collagen monomers to quickly precipitate into a whitish, amorphous mass. In addition, overmixing—even gently—can cause the collagen to polymerize rapidly into clumps of short fibrils, resulting in a weak, paste-like gel of diminished clarity. The tendency for premature polymerization, precipitation, and clumping of collagen fibrils is increased in solutions that are excessively basic; therefore, it is important to adjust the pH to no higher than 7.2.
4. *Fabrication of nylon mesh rings*: As mentioned in Subheading 3.5, small rings of nylon mesh are used to support the collagen gel. Custom-made rings are prepared commercially either by

cold-stamping with a sharp-edged punch or by laser-cutting. In our experience, we prefer cold-stamped rings to laser-cut rings, as the former have clean, incised edges, whereas the latter have melted edges of greater irregularity. Commercially made rings are relatively expensive, but provide a highly uniform product and save the investigator the time required for fabrication by hand. However, to limit expense, rings can be cut out of bulk nylon mesh (available from Sefar America or McMaster-Carr, Inc., Santa Fe Springs, CA) using disposable 3- and 6-mm biopsy punches (Thermo Fisher Scientific, Inc., Waltham, MA) for the inner and outer diameters, respectively. Alternatively, small rectangular frames can be cut out from bulk mesh using hobby knives or #11 scalpel blades and printed paper templates generated with Microsoft PowerPoint or Adobe Photoshop.

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