Published in final edited form as:

Methods Mol Biol. 2014 ; 1135: 393–402. doi:10.1007/978-1-4939-0320-7_32.

Assays to Examine Endothelial Cell Migration, Tube Formation, and Gene Expression Profiles

Shuzhen Guo, Josephine Lok, Yi Liu, Kazuhide Hayakawa, Wendy Leung, Changhong Xing, Xunming Ji, and Eng H. Lo

Abstract

Common methods for studying angiogenesis in vitro include the tube formation assay, the migration assay, and the study of the endothelial genome. The formation of capillary-like tubes in vitro on basement membrane matrix mimics many steps of the angiogenesis process in vivo and is used widely as a screening test for angiogenic or antiangiogenic factors. Other assays related to the study of angiogenesis include the cell migration assay, the study of gene expression changes during the process of angiogenesis, and the study of endothelial-derived microparticles. Protocols for these procedures will be described here.

Keywords

Angiogenesis; Endothelial cells; Matrigel matrix; Tube formation; Migration; Transcriptome; Microparticles

1 Introduction

The study of a complicated biological process such as angiogenesis requires the use of appropriate in vitro models to facilitate an understanding of the signaling pathways in angiogenesis. About 30 years ago, Montesano and Orci reported that bovine microvascular endothelial cells formed tubelike structures in a 3D collagen-I gel and that this process may serve as an in vitro angiogenesis model [1]. This model was expanded by George E. Davis to represent vasculogenesis or angiogenesis [2]; however, the assay required a period of several days, and was limited by the fact that not all the cells formed tubes, making it difficult to perform any type of quantification. In 1988 Kubota et al. showed that the use of a reconstituted basement membrane matrix enabled endothelial cells to attach and align rapidly and to form capillary-like tubes within hours, representing many of the steps of angiogenesis in vivo. These capillary-like tubes had characteristics of endothelial cells in vivo, including the ability to form tight cell-cell contacts and to take up acetylated low-density lipoprotein [3]. The basement membrane matrix tube formation assay has been shown to be an easy, rapid, and reliable assay and is widely used to study the signaling pathways and mechanisms of angiogenesis, along with migration assays and proliferation assays [4].

Additional important assays for the study of endothelial cells include the analyses of the endothelial genome as well as the study of endothelial vesicles. A large amount of vesicles are found to originate from eukaryotic cells and are present in almost all body fluids [5]. These vesicles are called exosomes, microparticles, or microvesicles, according to their origins and characteristics [6]. These vesicular structures are pivotal in cellular communication both under physiological and pathological conditions [7, 8]. For instance, extracellular vesicles derived from platelets are reported to promote angiogenesis [9, 10]. Likewise, endothelial-derived microparticles containing proteins or mRNA of the matrix metalloproteinase family were also reported to contribute to angiogenesis [11, 12].

In this chapter we describe the Matrigel tube formation assay, the scratch migration assay [13], the transcriptome analysis of brain microvascular endothelial cells purified by PECAM-1 antibody [14], and the preparation of extracellular vesicles, including microparticles and microvesicles.

2 Materials

2.1 Growth Factor Reduced (GFR) Matrigel Matrix (BD Bioscience, San Jose, CA)

Matrigel is the most commonly used commercial basement membrane. A solubilized matrix extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, it contains laminin, collagen IV, entactin, and heparin sulfate proteoglycan (perlecan). GFR BD Matrigel matrix contains reduced concentrations of growth factors compared to standard Matrigel matrix, resulting in a lower basal level of tube formation, which is advantageous for testing factors that increase angiogenesis.

2.2 Rat Brain Endothelial Cells

1. The rat brain microvascular endothelial cell line, RBE4.
2. Endothelial growth medium (EGM), as endothelial basal medium (EBM-2) with supplements (catalogue # CC-4147, Lonza, Cambridge, MA), which consist of 5 mL fetal bovine serum, hEGF, hydrocortisone, GA-1000 (gentamicin, amphotericin-B), VEGF, hFGF-B, R3-IGF-1, and ascorbic acid. The working medium is EBM-2 with 100 units/mL penicillin-streptomycin without serum and any supplements.
3. RBE4 cells were plated on collagen-I-coated flasks or plates.

2.3 Cell Detachment Solution

0.05 % trypsin-EDTA solution, stored at 4 °C.

2.4 Fixation Solution

4 % paraformaldehyde (PFA).

2.5 Animals for Brain Endothelial Cell Preparation

CD57 mice (6–8 weeks old) were used. For each preparation pool the cortexes from 3 mice.

2.6 Reagents for Endothelial Cell Purification

1. HBSS without calcium/magnesium, referred to as HBSS in this chapter.
2. Collagenase-A dissolved in H₂O and stored as 200 mg/mL stock in −20 °C. The working solution is 2 mg/mL diluted in HBSS with calcium/magnesium and warmed to 37 °C for use.
3. Rat antibody anti-mouse PECAM-1 (clone MEC13.3, Pharmingen, Franklin Lakes, NJ).
4. Dynabeads M-450 with sheep anti-rat IgG (DynaL Biotech).
5. Magnetic separator (DynaL Biotech).

2.7 Reagents for Total RNA Preparation and Measurement

1. 14.3 M β-mercaptoethanol (β-ME).
2. Ethanol (70, 80, and 96–100 %), molecular reagent grade.
3. RNeasy Plus Micro Kit (Qiagen).
4. RNA 6000 Pico kits including reagents and chips (Agilent Technologies, Santa Clara, CA).
5. Ovation Pico WTA system (NuGEN, San Carlos, CA).

2.8 Exosome-Free Fetal Bovine Serum (FBS)

The FBS used for conditioned culture medium to prepare extracellular vesicles is prepared by ultracentrifugation at 100,000 × *g* for 16 h prior to use, to remove bovine exosomes.

3 Methods

3.1 Matrigel Tube Formation

1. Thaw the Matrigel on ice or in cold room completely and slowly (*see* Notes 1 , 2). Keep it on ice until use.
2. Keep the multiwell plate on ice. Use precooled tips to add 100 μL soluble matrix into wells of 48-well plate, making sure that it covers the well evenly, with no bubbles in the gel mixture.
3. Incubate the plates with Matrigel at 37 °C for 30–60 min. This process causes the matrix to polymerize into insoluble gels. The Matrigel is now ready for use.
4. Detach RBE4 cells with detachment solution when they reach 80–90 % confluence. Collect and suspend the cells in EBM-2 with 0.5 % FBS.

¹It is recommended to use BD Matrigel matrix protein concentrations of 10 mg/mL or greater. Because the effect of Matrigel from different lots on tube formation may be different, it is preferable to use Matrigel from the same lot number for the entire experiment.

²Thaw the soluble matrix slowly on ice, usually over night. Once thawed, swirl the bottle slowly on ice to make the solution even. Avoid repeated freezing and thawing. The unused Matrigel matrix should be dispensed into appropriate aliquots and refrozen immediately.

5. Count the cells with a hemocytometer, diluting the cells in EBM-2 with 0.5 % FBS to 2.5×10^4 per cells/mL (*see* Note 3).
6. Seed the diluted RBE4 cells on to the surface of Matrigel-coated wells at a density of 5×10^3 cells/well (*see* Note 4).
7. Incubate the plate in the CO₂ incubator for 6–18 h, with temperature at 37 °C (*see* Note 5).
8. Examine the cells using a phase-contrast microscope, and take 2–3 images for each well using the light microscope (*see* Note 6).
9. Count the number of complete rings in each well. The number of rings is a measure of the capability to form tubes and a reflection of the angiogenic ability (*see* Note 7).

3.2 Scratch Migration Assay

1. Use RBE4 cells when confluent, wash twice with PBS, then add 3 mL cell detachment solution, and incubate at 37 °C for 5 min.
2. Add fresh 3 mL EGM, gently pipette to resuspend the cells, and centrifuge the suspension to get a cell pellet.
3. Resuspend the cell pellet with EGM, and plate 2×10^5 cells per well in a 6-well plate pre-coated with collagen-I or 5×10^4 cells per well in a 24-well plate.
4. Return the plates to the CO₂ incubator at 37 °C. Change to fresh EGM every 2–3 days until the cells are almost confluent (*see* Note 8).
5. Change to working medium, and incubate overnight for serum starvation (*see* Note 9).
6. Scrape the cell monolayer in a straight line with a p200 or p1000 pipette tip (*see* Note 10). Remove the debris by washing twice with warm working media, and then replace with fresh working medium.

³The angiogenic or antiangiogenic factors for testing can be added into the cell suspension before seeding.

⁴The cell number seeded in each well is critical for this assay. Using an inadequate number of cells yields incomplete tubes, while using too many cells yields large areas of monolayers. The optimum cell number is suggested to be approximately 4,800 cells/cm²; however, this number may vary with different types of endothelial cells [4].

⁵The cells could elongate and align to each other as early as 3 h after seeding. The images could be acquired at an early time (~6 h incubation time) or late time (~18 h incubation time) for analysis [4]. After longer incubation times, the proteinases secreted by the cells may digest the gels and destroy the supports for tubes.

⁶For the thin coating of Matrigel matrix described here, the surface is not flat but crescent, which may make it difficult to get photos with good focus. There is a μ -Slide Angiogenesis chamber from ibidi GmbH (Planegg/Martinsried, Germany) used with only 10 μ L of matrix and a flat surface to bring all cells into one focal plane.

⁷The parameters for quantitative image analysis could be numbers, areas or perimeters of rings (tubes), or the numbers of branching points.

⁸To maintain the cells in a monolayer, the endothelial cells should not be overgrown. Overgrown cells could be arranged in multiple layers, which cause higher amount of cell debris or more free-floating cells after scratching.

⁹Serum starvation is an important step to minimize background signal before cell stimulation.

¹⁰To create scratches of approximately similar size, it is critical to minimize any possible variation caused by a difference in the width of the scratches. For 24-well plates, the p200 pipette tip can be used more easily than the p1000 pipette tip.

7. To enable imaging the area with scratch injury, mark the location of the scratch with reference points on the outer bottom of the culture plate using a razor blade or an ultrafine tip marker.
8. Return the cells back to the CO₂ incubator for 8–24 h (*see* Note 11).
9. After the incubation, wash the cells twice with PBS gently to remove detached or dead cells, and fix cells with 4 % PFA for 10 min at 4 °C (*see* Note 12).
10. Place the culture plate under a phase-contrast microscope, locate the scratch injury site using the reference point, and acquire images from at least 4 random areas (*see* Note 13).
11. Quantify the number of migrated cells by counting cells that cross into the scratched area from their reference points (*see* Note 14).

3.3 The Transcriptome of Mouse Brain Microvascular Endothelial Cells (See Note 15)

3.3.1 Preparation of Anti-Mouse PECAM-1 Dynabeads—

1. Take 12 µL of bead slurry; mix well in a 15 mL tube with 0.1 % BSA in PBS (*see* Note 16).
2. Place the tube in a magnetic separator for 1–2 min, then remove the supernatant.
3. Repeat washing as above for 3 times.
4. Resuspend the beads in 100 µL of 0.1 % BSA in PBS, then add 1 mL HBSS.
5. Add the rat anti-mouse PECAM-1 into the beads solution, at the ratio of 1 µg antibody for every 40 µL of beads. Incubate overnight on a rotator at 4 °C (or 2 h at RT) for antibody linkage.
6. Wash the beads/antibody mix with 2 mL 0.1 % BSA in PBS, for 3 times.
7. Resuspend the beads in original volume of 12 µL 0.1 % BSA to maintain beads at 4×10^8 beads/mL. Store the beads at 4 °C and they are ready for the purification. Use within 1–2 weeks.

3.3.2 Tissue Dissection and Dissociation—

¹¹Choose shorter incubation times under faster migrating conditions.

¹²Before imaging, the washing step is important. Wash gently to remove all debris and free-floating cells to distinguish real migrating cells. Fixing cells helps to keep cell morphology and allows a longer imaging time.

¹³RBE4 cells migrate into the scratched area under basal conditions (with working medium). Four random areas from both edges of the scratched area should be photographed, providing large sample sizes for statistical analysis.

¹⁴Acquired images could be further analyzed quantitatively with other indexes, such as the migrating distance from reference point of at least 100 cells for each condition, or the area occupied by migrated cells. Some free software are available, such as Image J (<http://rsb.info.nih.gov/ij/>).

¹⁵Great care should be taken during the preparation to eliminate possible RNase contamination and during the whole procedure to avoid introducing RNase into the samples. The use of sterile, disposable plastic wares (dishes and tubes) is recommended throughout the procedure. The reusable instruments should be cleaned and presoaked in 0.1 % DEPC for at least 12 h, rinsed with DW water, and autoclaved before use. Prepare all solutions using RNase-free water. Always wear latex or vinyl gloves while handling reagents and samples and change gloves frequently.

¹⁶Prepare 4 µL of beads for each brain.

1. Sacrifice the mice by intracardiac perfusion with PBS, and wash the body with a generous amount of 70 % ethanol.
2. Harvest the brains in HBSS on ice; dissect the brains to remove the cerebral cortex.
3. Roll the cortical tissue on 3MM filter paper (autoclaved) to remove the big vessels.
4. Transfer the tissue to a new dish with a small amount of HBSS and finely mince the tissue with scissors.
5. Transfer the minced tissue to a 50 mL tube with warm collagenase-A solution (*see* Note 17).
6. Incubate the tissue in collagenase solution at 37 °C with gentle shaking for 20 min.
7. Using 14 gauge catheters attached to a syringe, triturate the enzyme suspension until there are no obvious big pieces.
8. Filter the enzyme suspension through a 70 µm cell strainer into a new 50 mL tube.
9. Centrifuge the cell suspension at 4 °C, 400 × *g* for 8 min.
10. Resuspend the pellet with 10 mL HBSS, centrifuge again for washing.

3.3.3 Purification of Endothelial Cells—

1. Resuspend the pellet with 4 mL HBSS, and transfer into a new 15 mL tube.
2. Add anti-PECAM-1-coated Dynabeads, and mix by carefully rotating the tube up and down.
3. Incubate at room temperature for 10 min, or at 4 °C for 30 min, with gentle rotation for binding of endothelial cells to anti-PECAM-coated Dynabeads.
4. Mount the tube on a magnetic separator and wait for 1–2 min; then remove the supernatant (*see* Note 18).
5. Remove the tube from the magnetic separator and resuspend the Dynabeads with 4 mL HBSS by vigorous trituration (*see* Note 19).
6. Repeat **steps 4, 5** for 3 times, then remove the supernatant.
7. After the last wash, directly add 400 µL Buffer RLT plus (with 1 % β-ME) for RNA preparation or freeze the Dynabead-bound cells in –80 °C for later preparation.

3.3.4 Preparation of RNA from Purified Endothelial Cells—Following the protocol suggested by the manufacturer:

1. Transfer the lysate of endothelial cells with Dynabeads to a 1.5 mL Eppendorf tube.

¹⁷HBSS with calcium/magnesium is used to make collagenase-A solution, since the enzyme needs cations for its function. HBSS without calcium/magnesium is used in other steps, especially for the steps with magnetic beads.

¹⁸The cell suspension here is cloudy; more HBSS can be added before placing the tube on the magnetic separator, making it easier to remove the supernatant while not losing beads.

¹⁹Triturate vigorously here to wash out the contamination of other types of cells.

2. Homogenize the cell lysate with a 20 gauge needle attached to syringe, passing the cell lysate through the needle several times.
3. Transfer the lysate to the gDNA Eliminator spin column and centrifuge at $10,000 \times g$ for 30 s.
4. Add 400 μ L 70 % ethanol to the flow-through and mix well by gently pipetting.
5. Transfer the mixture to an RNeasy MinElute spin column in collection tube, and centrifuge at $10,000 \times g$ for 30 s. Discard the flow-through.
6. Wash the RNeasy MinElute spin column with 700 μ L Buffer RW1 and 500 μ L Buffer RPE, respectively, by centrifuge at $10,000 \times g$ for 30 s.
7. Wash again with 500 μ L 80 % ethanol at $8,000 g$ for 2 min. And completely dry the column by centrifuge in a new collection tube at maximum speed for 5 min.
8. Transfer the column to a new collection tube; elute the RNA sample with 30 μ L H_2O . Aliquot the RNA samples on ice and store at $-80^\circ C$.

3.3.5 Quantification and Qualification of RNA Samples for Microarray Service

1. Check the quantity of RNA samples. Apply 1 μ L of RNA onto the NanoDrop, for quantification of RNA concentration, and the ratio of A_{260}/A_{280} and A_{230}/A_{280} .
2. Check the integrity of the RNA samples with Agilent 2100 Bioanalyzer. Apply 1 μ L of RNA (diluted to concentrations less than 1 ng/mL) to Eukaryotic RNA Pico chip following the protocol of manufacturer. The respective ribosomal RNAs should appear as sharp peaks. The apparent ratio of 28S rRNA to 18S rRNA should be approximately 2. The RIN (RNA integrity number) score should be larger than 7.0.
3. Use 500 pg–50 ng of RNA in a volume less than 5 μ L for amplification to get cDNA with the NuGEN Ovation Pico WTA system, then fragmentation and labeling with Encore Biotin Module. The individual samples are hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 microarray.

3.4 Preparation of Extracellular Vesicles

1. Use cells at 70 % confluence in several 15 cm culture dishes (*see* Note 20).
2. Discard culture media and wash thoroughly with pre-warmed PBS three times (*see* Note 21).
3. Add culture media with exosome-free FBS, and incubate 24–48 h to collect conditioned media with extracellular vesicles (*see* Note 22).

²⁰Media used for culture should be at room temperature before using. If unclear whether there is an adequate amount of extracellular vesicles production, collect as much conditioned culture medium as possible.

²¹Cells should be washed thoroughly with PBS, especially if serum deprivation is required. This removes any residual nucleic acid and proteins from the serum.

²²Extracellular vesicle harvesting time varies from 24 to 48 h, depending on cell confluence.

4. Collect the conditioned medium and centrifuge at $300 \times g$, 4°C for 10 min, and followed by $2,000 \times g$, 4°C for 20 min (*see* Note 23).
5. (a) To get microparticles, further centrifuge the supernatant at $20,000 \times g$, 4°C for 20 min, resulting in a pellet containing the microparticles (*see* Note 24). (b) To get microvesicles, filter the supernatant with a $0.2\ \mu\text{m}$ filter, and then ultracentrifuge at $100,000 \times g$ (Rotor 70Ti) for 2 h, which results in a pellet containing the microvesicles (*see* Note 25).
6. Wash the pellets with filtered PBS, then resuspended in filtered PBS, and store at -80°C (*see* Note 26).

References

1. Montesano R, Orci L. Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell*. 1985; 42(2):469–477. [PubMed: 2411423]
2. Koh W, Stratman AN, Sacharidou A, Davis GE. In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis. *Methods Enzymol*. 2008; 443:83–101. [PubMed: 18772012]
3. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol*. 1988; 107(4):1589–1598. [PubMed: 3049626]
4. Arnaoutova I, George J, Kleinman HK, Benton G. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis*. 2009; 12(3):267–274. [PubMed: 19399631]
5. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One*. 2012; 7(3):e30679. [PubMed: 22427800]
6. Le Bihan MC, Bigot A, Jensen SS, Dennis JL, Rogowska-Wrzesinska A, Laine J, Gache V, Furling D, Jensen ON, Voit T, Mouly V, Coulton GR, Butler-Browne G. In-depth analysis of the secretome identifies three major independent secretory pathways in differentiating human myoblasts. *J Proteomics*. 2012; 77:344–356. [PubMed: 23000592]
7. Lai CP, Breakefield XO. Role of exosomes/microvesicles in the nervous system and use in emerging therapies. *Front Physiol*. 2012; 3:228. [PubMed: 22754538]
8. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008; 10(12):1470–1476. [PubMed: 19011622]
9. Kim HK, Song KS, Chung JH, Lee KR, Lee SN. Platelet microparticles induce angiogenesis in vitro. *Br J Haematol*. 2004; 124(3):376–384. [PubMed: 14717787]
10. Shai E, Varon D. Development, cell differentiation, angiogenesis–microparticles and their roles in angiogenesis. *Arterioscler Thromb Vasc Biol*. 2011; 31(1):10–14. [PubMed: 21160063]
11. Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Pathol*. 2002; 160(2):673–680. [PubMed: 11839588]
12. Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G. Endothelial progenitor cell derived microvesicles activate an angiogenic program in

²³During the preparation of vesicular structures, use 4°C prechilled solutions. The centrifuge should be performed sequentially and immediately; frozen-thaw of conditioned medium even once would lead to a lot of unexpected pellets.

²⁴For microparticle preparation from blood, platelets can be easily stimulated to secrete more microparticles and can be an additional source of microparticles. It is highly recommended that one uses platelet-free plasma, along with citrate sodium as an anticoagulant.

²⁵Pure exosomes can be further purified by using continuous sucrose gradients.

²⁶The filtered PBS here should be prepared freshly and filtered through a $0.2\ \mu\text{m}$ filter, for washing and dissolving the extracellular vesicles pellets.

- endothelial cells by a horizontal transfer of mRNA. *Blood*. 2007; 110(7):2440–2448. [PubMed: 17536014]
13. Xing C, Lee S, Kim WJ, Wang H, Yang YG, Ning M, Wang X, Lo EH. Neurovascular effects of CD47 signaling: promotion of cell death, inflammation, and suppression of angiogenesis in brain endothelial cells in vitro. *J Neurosci Res*. 2009; 87(11):2571–2577. [PubMed: 19360900]
 14. Guo S, Zhou Y, Xing C, Lok J, Som AT, Ning M, Ji X, Lo EH. The vasculome of the mouse brain. *PLoS One*. 2012; 7(12):e52665. [PubMed: 23285140]