Chapter 6

The Aortic Ring Assay and Its Use for the Study of Tumor Angiogenesis

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Abstract

This chapter describes protocols developed in our laboratory to prepare and analyze angiogenic cultures of rat aorta. Rings of rat aorta cultured in collagen gels produce neovessel outgrowths which reproduce ex vivo key steps of the angiogenic process including endothelial migration, proliferation, proteolytic digestion of the extracellular matrix, capillary tube formation, pericyte recruitment, and vascular regression. The angiogenic response of aortic explants can be stimulated with growth factors or inhibited with anti-angiogenic molecules. Aortic ring cultures can also be used to study tumor angiogenesis. Protocols outlined in this chapter describe how this assay can be modified to investigate the angiogenic activity of cancer cells.

Key words Collagen, Endothelial cells, Pericytes, Neovascularization, Cancer cells

1 Introduction

Angiogenesis contributes to the progression of many debilitating and life-threatening diseases but it also plays a critical role in embryonal development, the revascularization of ischemic organs, and the regeneration of tissues [1]. Basic research conducted during the last 40 years has greatly enhanced our understanding of how blood vessels form, mature, and remodel and has uncovered key molecular pathways of angiogenic regulation that can be targeted pharmacologically for therapeutic intervention [1]. Successful translational studies and clinical trials have led to the approval for therapeutic applications of many angiogenesis-targeting drugs [2, 3]. Research in this field has created the need for bioassays to test the activity of pro- and anti-angiogenic compounds. Among the many systems that have been developed to study angiogenesis [4], the aortic ring model has emerged as one of the most widely used assays particularly for the evaluation of new drugs. A Medline review shows that nearly 500 studies have been published to date

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with the aortic ring assay and that its use has steadily increased in recent years [5].

The aortic ring assay recapitulates ex vivo the main steps of the angiogenic process including endothelial migration, proliferation, proteolytic digestion of the extracellular matrix, capillary tube formation, recruitment of pericytes, and vascular regression [6]. Except for the lack of blood flow, the angiogenic response of the aortic explants resembles angiogenesis in vivo and is regulated by paracrine interactions between endothelial cells, macrophages, fibroblasts, and pericytes involving a variety of angiogenic factors and inflammatory cytokines/chemokines [5].

In this chapter we provide a detailed description of protocols currently used in our laboratory to prepare aortic ring cultures and analyze angiogenesis in this model. We also describe how this assay can be used to study tumor angiogenesis.

2 Materials and Reagents

1. 1–2-month-old rat.
2. Styrofoam dissecting board.
3. 80% Ethanol.
4. Hair clippers with size 40 A5 blade.
5. Size 2.0 silk sutures.
6. One pair of 17 cm curved Mayo scissors.
7. Two Halsted-Mosquito hemostats.
8. Size 22 surgical blades and scalpel blade handle.
9. Small forceps.
10. Small curved scissors.
11. 100 mm four-compartment Felsen dishes.
12. Four-well tissue culture dishes (NUNC—IVF dishes).
13. Endothelial Basal Medium (EBM, Lonza).
14. Rat tail collagen solution (1.3 mg/ml, either commercial or prepared in-house).
15. Fine-curved microdissection forceps (Dumont #7).
16. 14 mm straight blade Noyes iridectomy scissors.
17. Sterile 23.4 mg/mL sodium bicarbonate solution.
18. Sterile 10X minimum essential medium (MEM) solution.
19. Micropipettors and sterile tips.
20. Humidified 5% CO_2 tissue culture incubator.
21. Dissecting microscope.

3 Methods

3.1 Excision of the Aorta

1. Sacrifice rats by CO_2 asphyxiation or intraperitoneal injection of sodium pentobarbital.
2. Shave thoracic and abdominal skin using hair clippers, and disinfect with 80% ethanol.
3. Pin the animal onto a dissection board.
4. Make a Y-shaped incision with a scalpel from the top of each shoulder to the xyphoid process of the sternum and down to the lower abdomen.
5. Dissect the skin from the underlying muscle with a scalpel and open the abdominal cavity using small scissors.
6. Cut the ribs with scissors from each side of the animal toward the manubrium of the sternum. Using a hemostat, gently displace the sternal plate to the right side of the animal exposing the thoracic cavity.
7. Using sterile forceps, displace the intestines, stomach, spleen, and liver to the right side of the animal.
8. Cut the diaphragm with sterile small curved scissors toward the midline and back, avoiding the diaphragmatic vessels: this will expose the thoracic aorta along the vertebral column.
9. Tie a knot with a silk suture at the distal end of the thoracic aorta, just below the diaphragm. While holding the suture with forceps, dissect the aorta from the posterior mediastinum with small curved scissors. Excise the aorta at the aortic arch level.
10. Cut the aorta below the knot and place it in a Felsen dish containing serum-free EBM.

3.1.1 Notes

1. We recommend using the thoracic portion of the aorta which has a uniform diameter and a regular pattern of collateral (intercostal) arteries. The abdominal artery (below the diaphragm) is an additional source of rings, but its tapering lumen may introduce variability in the angiogenic response.
2. Attention should be given not to damage the aorta by stretching or letting it dry. The isolation procedure should be completed within 10–15 min of animal sacrifice.

3.2 Preparation of Aortic Rings

1. Working under a dissecting microscope, clean the aorta of periaortic fibroadipose tissue and blood using curved microdissection forceps and Noyes iridectomy scissors.
2. Remove the intraluminal blood clot using fine microdissection forceps.
3. Cut away the collateral vessels as close to the aorta as possible.

4. As the cleaning proceeds, transfer the aorta into the adjacent compartments of the Felsen dish, always ensuring that the aorta is submerged in fresh medium.
5. Prepare aortic rings by serially cross-sectioning the aortic tube at 1–1.5 mm intervals with a scalpel. Discard the proximal and distal rings.
6. Wash the rings through eight sequential baths of serum-free EBM using two Felsen dishes. Lift and hold rings between washes using the capillary action of medium trapped between the microdissection forceps prongs.
7. After washing, re-examine rings to ensure that they are uniform in size. Trim off any remaining fibroadipose tissue. The aortic rings are now ready to be embedded in collagen gels.

3.2.1 Notes

1. The thoracic aorta of a 2-month-old rat should yield 20–25 rings.
2. When cutting rings from the aorta with a scalpel, it is helpful to remove some of the growth medium from the chamber so that the aorta is kept wet but no longer floats.
3. When transferring rings between washes, take care not to crush or otherwise damage the rings.
4. The total time required to clean a rat aorta, cut individual rings, and perform eight washes should not take longer than 30–45 min.
5. Once aortic rings have been prepared, they may be stored in EBM in a humidified tissue culture incubator at 37 °C for periods of up to 24 h prior to embedding in collagen gels.

3.3 Embedding Aortic Rings in Collagen Gel

1. Prepare the working collagen solution by mixing 1 volume 10× MEM with 1 volume 23.4 mg/ml $NaHCO_3$ in a sterile tube. Next add eight volumes of 1.3 mg/ml collagen solution and mix by pipetting slowly up and down several times taking care not to generate bubbles. Keep all solutions on ice.
2. Transfer aortic rings with fine-curved microdissection forceps into four-well IVF dish. Place one ring on the bottom of each culture well.
3. Pipette a 30 μl drop of working collagen solution onto each ring.
4. Using a sterile pipette tip, reposition the aortic ring so that it is suspended in the collagen solution and oriented with the luminal axis parallel to the bottom of the culture dish.
5. Spread the collagen solution around the explant into a thin disc of approximately 8 mm in diameter by slowly drawing the collagen outward around the ring in a circular motion.

6. Incubate the IVF dish containing four aortic ring cultures into a tissue culture incubator at 37 °C for 10 min to induce collagen gelation.
7. Carefully add 500 μl of serum-free EBM to each culture in a dropwise fashion.
8. Replace growth medium with fresh medium three times a week starting from day 3.

3.3.1 Notes

1. This method is optimized for assaying angiogenic growth from collagen gel cultures of rat aortic rings. The same method can be applied to mouse aortic rings with minor modifications of the culture conditions [6–8].
2. Always prepare four aortic ring cultures per experimental group including an untreated set of cultures.
3. The collagen solution can be prepared from rat tail tendons as described [6, 9] or obtained from commercial sources [7].
4. Alternative matrices such as fibrinogen or Matrigel may be used, as needed [10–12].
5. The procedure should be carried out without interruptions, always keeping the aortic rings submerged in culture medium to avoid drying of the aortic explants.
6. It is important not to stretch or crush the aorta or the aortic rings at any stage of the procedure.
7. Always mix the 10× MEM and $NaHCO_3$ solutions thoroughly before adding the collagen solution.
8. Keep all reagents for the collagen gel on ice.
9. The collagen solution should not be less than 1.3 mg/ml dry weight and all reagents should be of tissue culture grade and LPS (endotoxin)-free. Aortic rings are very sensitive to LPS which can stimulate or inhibit angiogenesis depending on its concentration [13].
10. Before adding collagen to the aortic ring, remove excess medium carried over during transfer to the IVF dish by gently dragging the wet ring across the surface of the dish.
11. Neutral pH should be maintained throughout the culture period.
12. Control and experimental aortic rings should always be dissected from animals of the same age, ideally from the same aorta.
13. Feeding is not required if the cultures are not supplemented over time with exogenous agents. If the cultures are not fed, the angiogenic response of the rings is more pronounced and the aortic neovessels survive longer, likely due to accumulation of endogenous angiogenic factors in the culture medium.

3.4 Preparation of Cancer Cell Aggregates

1. Pipette 4 ml of a 1×10^6 suspension of cancer cells in EBM + 10 % FBS into a sterile 25 ml Erlenmeyer flask.
2. Place flask on an orbital shaker set at ~75 rpm, and culture for 48 h in a humidified incubator at 37 °C.
3. Look for clustering of cells and formation of aggregates in the middle of the flask.
4. Transfer to a 15 ml tube and let aggregates sediment by gravity to the bottom of the tube. If aggregates are too small (this will depend on the cancer cell type), centrifuge at low speed to facilitate sedimentation.
5. Remove supernatant, resuspend aggregates in 0.5 ml collagen solution (see above), and mix with a micropipettor P1000. Keep suspension of cancer cell aggregates in collagen solution on ice.

3.5 Co-culture of Aortic Rings with Cancer Cell Aggregates

1. Pipette 5 μl of collagen/cell aggregates onto bottom of a four-well IVF dish, and incubate at 37 °C for 5 min to induce gel formation. This will generate a collagen gel drop containing cancer cell aggregates.
2. Place an aortic ring next to the cancer cell/collagen gel drop, and pipette an additional 30 μl collagen solution onto the culture dish to cover both aortic ring and adjacent cancer cell/ collagen gel drop.
3. Use a pipette tip to ensure that the collagen gel is uniformly spread around the aortic explant and adjacent cancer cells.
4. Incubate for 10 min at 37 °C.
5. Add 500 μl serum-free EBM and incubate at 37 °C. Feed 3× a week.

3.6 Treatment of Aortic Ring Cultures with Tumor-Conditioned Medium

1. Prepare tumor-conditioned medium by incubating subconfluent cultures of cancer cells in serum-free EBM for 3 days. Use fresh or store at −70 °C.
2. Prepare aortic ring cultures as described above.
3. Feed the aortic ring cultures with a 1:1 mixture of fresh serum-free EBM and tumor-conditioned medium. Dose–response experiments can be performed by varying the ratio of fresh EBM and tumor-conditioned EBM.

3.6.1 Notes

1. For the co-culture experiments, place the ring within 1 mm from the cancer cell aggregates to ensure maximum exposure to tumor angiogenic factors.
2. The size of the cancer cell aggregates will vary depending on the cancer cell line.
3. If cancer cells have special media requirements, prepare tumor-conditioned medium with the cancer cell-specific medium, always without serum. Use this medium for the 1:1 mixture with EBM in the untreated control.

4 Measurement of Angiogenesis

Angiogenesis can be measured either by manually counting vessels with an inverted microscope or by image analysis. Image analysis provides an objective measurement of the angiogenic response including vessel number and length but it may be time consuming unless it is automated. For the method with Image J software used in our laboratory, refer to a previously published paper [14]. The manual counting method allows rapid assessment of angiogenic response from living cultures. This method only requires a trained observer and an inverted microscope. The speed and extent of the angiogenic response are analyzed by graphing the number of neovessels produced by individual cultures over time [15, 16]. A brief description of the manual method [6] is outlined below.

4.1 Measurement of Angiogenesis by Counting Neovessels

1. Use an inverted microscope with 4× to 10× magnification equipped with bright-field optics. Adjust the condenser diaphragm to obtain optimal contrast and depth of field.
2. Start by visualizing a landmark of the outgrowth and then count all the visible vessels in either a clockwise or a counterclockwise direction.
3. Identify microvessels by their greater thickness and cohesive pattern of growth compared to isolated cells.
4. Count each branch as a separate vessel; a Y-shaped branching vessel is therefore counted as three vessels; loops are counted as two vessels; for anastomosing vessels forming polygonal patterns, count each side of the polygon as a separate vessel.
5. Count microvessels every 2–3 days and record the results for a period of 10–14 days.
6. Use statistical methods (*t*-test, analysis of variance) to analyze data and determine levels of significance between control and treated cultures.

4.1.1 Notes

1. Beware of adventitial collagen fibers or contaminating extraneous fibers which may mimic vessel sprouts.
2. The three-dimensional nature of the angiogenic growth requires frequent readjustment of the focus to accurately visualize vessels.
3. Vessel counts should be done by the same observer throughout the experiment.
4. The angiogenic outgrowth of a typical unstimulated collagen gel culture of rat aorta contains 60–100 microvessels.
5. As vessel number exceeds 200 per culture (cultures treated with exogenous angiogenic stimuli) the visual count method may become challenging and labor intensive. Since the outgrowths in these cultures are generally symmetrical, counting microves-

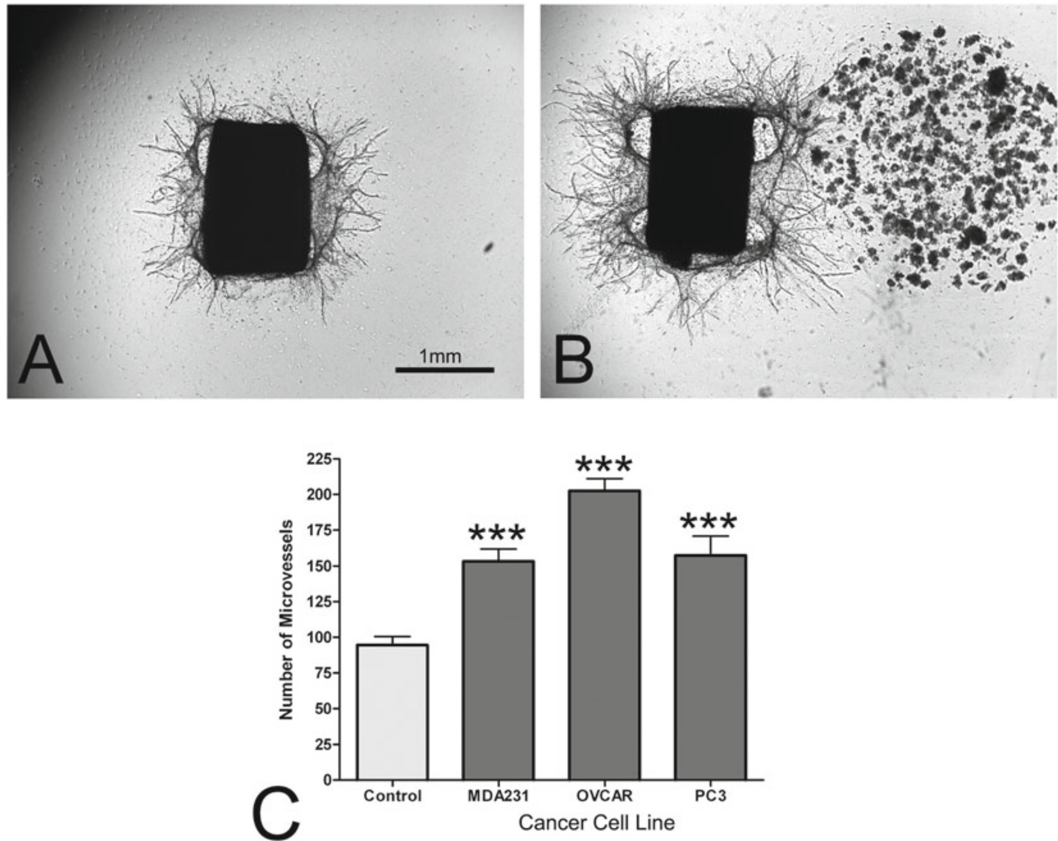

Fig. 1 Co-culture of aortic rings and cancer cell aggregates. (**a**) Angiogenic sprouting from aortic ring in control culture. (**b**) Angiogenesis stimulated by cancer cells in co-culture of aortic ring with aggregates of PC3 cancer cells. (**c**) Measurement of tumor angiogenesis in co-cultures of aortic rings with breast (MDA231), ovarian (OVCAR), and prostate cancer (PC3) cell lines ($N = 4$; $^{***}p < 0.001$)

sels in half the culture and then doubling the number will give a close approximation of the angiogenic response. Alternatively these cultures can be measured by image analysis [14].

6. We do not recommend the manual vessel count method if the aortic rings are cultured in Matrigel due to the complexity and highly branched nature of the outgrowths in this matrix. Angiogenesis in Matrigel is best visualized by image analysis after the cultures have been stained with endothelial specific markers.
7. Phase contrast is an acceptable alternative if sufficient contrast cannot be achieved with bright-field optics.

5 Expected Results

Most of the neovessels in aortic cultures originate from the cut edges of the explants. Sprouting endothelial cells form branches, anastomoses, loops, and networks which become coated with pericytes

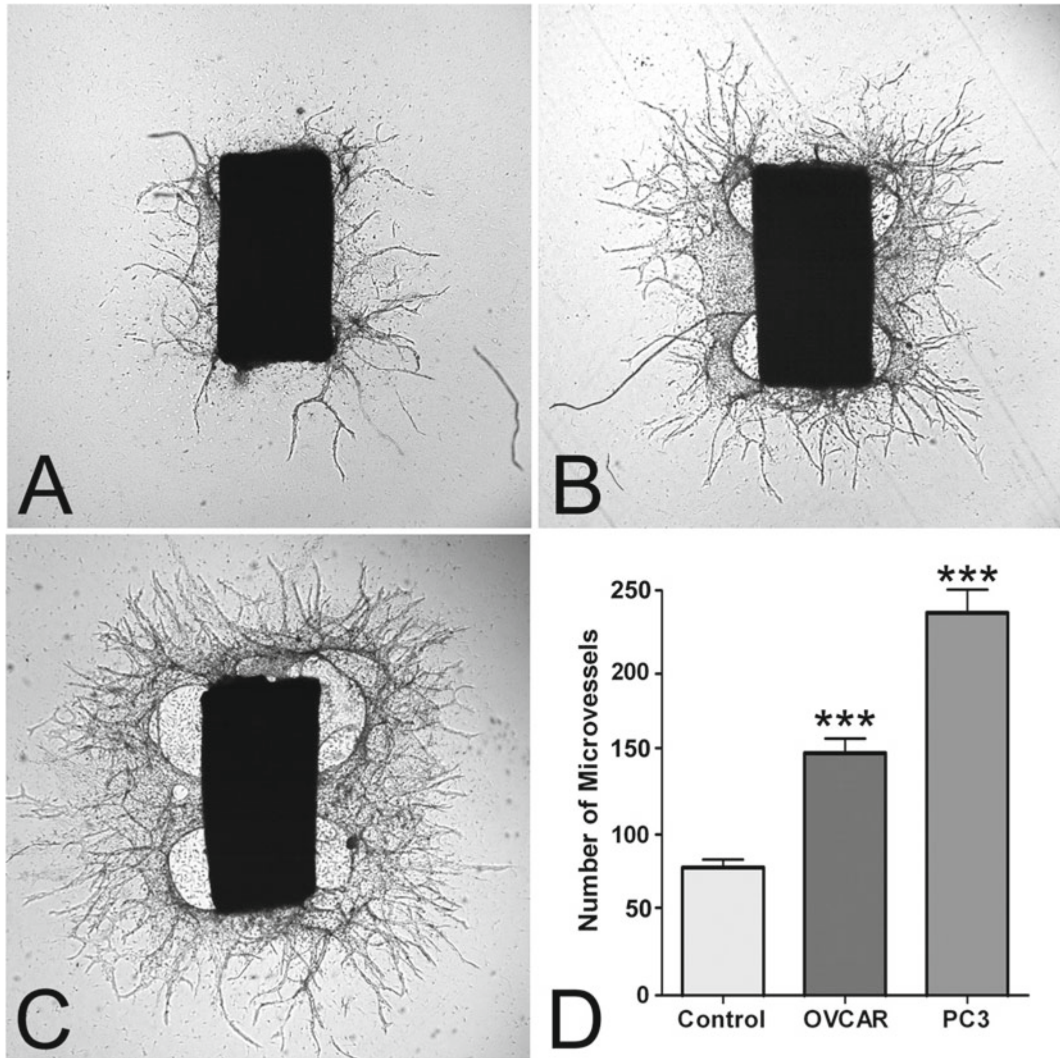

Fig. 2 Cultures of aortic rings stimulated with tumor-conditioned media. Control culture (**a**) and cultures treated with conditioned media from OVCAR (**b**) or PC3 (**c**) cancer cells. (**d**) Measurement of tumor angiogenesis in aortic ring cultures treated with tumor-conditioned media ($N = 4$; *** $p < 0.001$)

over time [5, 6, 9]. Endothelial sprouts become visible at days 2–3 and increase in number and length over the next 4–5 days. Angiogenic sprouting stops after 6–8 days of culture and is followed by vascular regression. Aortic angiogenesis can be greatly enhanced by exposing the cultures to endothelial growth stimuli including recombinant growth factors/cytokines or angiogenic factor-producing cells or tissue explants [5, 6, 9]. Figures 1 and 2 show the angiogenic effect in aortic cultures of human prostate, breast, and ovarian cancer cells or media conditioned by these cancer cells. This modification of the assay can be used to investigate mechanisms of tumor angiogenesis and test the efficacy of drugs designed to interfere with angiogenesis in different types of malignancy.

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