Chapter 2
Endothelial Cell Tube Formation on Basement Membrane to Study Cancer Neoangiogenesis

Amelia Casamassimi, Filomena de Nigris, Concetta Schiano, and Claudio Napoli

2.1 Introduction

During angiogenesis, endothelial cells (ECs) undergo activation after binding of angiogenic factors to their receptors, release of proteases to dissolve the basement membrane, migration towards an angiogenic signal, proliferation, and an increase in cell number for new blood vessel formation. Finally, reorganization of ECs forms the three-dimensional vasculature. Tube-formation assay is one of the simple, but well-established in vitro angiogenesis assays based on the ability of ECs to form three-dimensional capillary-like tubular structures, when cultured on a gel of growth factor-reduced basement membrane extracts. During the assay, ECs differentiate, directionally migrate to align, branch, and form the tubular polygonal networks of blood vessels.

A. Casamassimi (✉) • F. de Nigris
Department of Biochemistry, Biophysics and General Pathology,
Second University of Naples, 80138 Naples, Italy

C. Schiano
Institute of Diagnostic and Nuclear Development (SDN), IRCCS, 80143 Naples, Italy

C. Napoli
Department of Biochemistry, Biophysics and General Pathology,
Second University of Naples, 80138 Naples, Italy

Institute of Diagnostic and Nuclear Development (SDN), IRCCS, 80143 Naples, Italy

U.O.C. Division of Immunohematology, Transfusion Medicine and Transplantation Immunology (SIMT) and Regional Reference Laboratory of Immunogenetics and Transplantation Immunology (LIT), Second University of Naples, Naples, Italy

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The in vitro formation of capillary-like tubes by endothelial cells on a basement membrane matrix is a powerful in vitro method to screen for pro-angiogenic and anti-angiogenic factors. This assay can be used as a first screen before in vivo models and can be done also as a high throughput procedure. Since the first description in 1988, this assay have been used for many purposes both in cancer and cardiovascular fields, including analysis of pro-angiogenic or anti-angiogenic factors, definition of the signaling pathways involved in angiogenesis, identification of the genes regulating angiogenesis and endothelial progenitor cell characterization.

In our experimental settings we used in a model of osteosarcoma this assay to analyze the role of YY1 transcription factor and some cyclin-dependent kinases (CDK) on angiogenesis; to this aim, we performed tube formation assay to test both the effect of YY1 silencing and some inhibitors, like T22, a peptide affecting the chemokine receptor CXCR4 and roscovitine, a CDK inhibitor [1, 2].

2.2 Methodology

This angiogenesis assay consists of a quick measurement of the ability of ECs to form three-dimensional structures (tube formation) in vitro when they are plated on an appropriate extracellular matrix support. Endothelial tube formation on ECM gel mimics the in vivo environment and may be employed to test angiogenesis stimulators or inhibitors before in vivo analysis. Factors to be tested can be added exogenously to the medium, or they can be transfected or knocked down in the endothelial cells to determine their effects on angiogenesis.

As extracellular matrix support, the basement membrane extract obtained from a murine tumor rich in extracellular matrix proteins is generally used for this assay. The extract is in a liquid state at 4 °C or lower temperatures whereas it forms a gel at 16–37 °C. The routinely used basement membrane extracts are sold commercially; examples are BD Matrigel, Cultrex BME and other Engelbreth–Holm–Swarm (EHS) extracts. It is generally recommended the use of growth factor-reduced material especially to study stimulators of angiogenesis. Importantly, variability in the tube-formation activity of different preparations purchased from different vendors has also been observed. Thus, when possible, the same preparation should be used for the entire study.

Endothelial cells (ECs) typically used for this assay are obtained from human umbilical vein (HUVECs) or human aorta (HAECs), but other cells, such as lines established from mice (SVEC4-10) and humans (HMEC-1) also work well. It should be considered that ECs have considerable organ- and tissue-specific heterogeneity that may affect their response to specific factors and the time required for tube formation in vitro. The cells should be of low passages if they are primary; for instance, HUVECs should not be passaged more than 10 times, HAECs work well until the 7th–8th passage. The cells should be 80% confluent and passaged the day

before the assay for optimum and consistent tube formation. The seeding density is another important point to consider with about 50,000 cells per cm^2 as recommended density for HUVECs and HAECs, but this number may vary depending on the cell source. Cells initially attach to the matrix, then migrate toward each other, align and finally form tubes. The time of the assay is very short for transformed cells compared to primary cells (3 h versus 16–20 h for primary cells), but should be determined for each EC type. Moreover, time is also dependent on the utilized matrix.

2.3 Materials (and Company Name)

Endothelial cell lines can be those with and without drug treatment or expressing the gene of interest.

2.3.1 Reagents

Growth factor-reduced BD Matrigel (BD Biosciences 354230)
Primary Human Aortic Endothelial Cell (HAEC) (Lonza CC2535)
Trypsin-EDTA
Endothelial Basal Medium-2 (EBM-2; Lonza, CC-3156)
EGM-2 SingleQuot BulleKit (Endothelial cell growth medium-2; Lonza, CC-3162)
Dulbecco's Phosphate-Buffered Saline, 1x
OPTI-MEM (Life technologies, Gibco 31985-047)
Cultrex Cell Staining Solution (Trevigen, 3437-100-01)
Methanol (Sigma, M3641)
96-well cell culture plates
15 ml conical centrifuge tubes-sterile
Tissue culture flasks, 25 cm^2, filter cap, 50 ml
Disposable sterile plastic pipettes
ImageJ software downloaded from the NIH website.

2.3.2 Equipment

Tissue culture setup
Inverted microscope with digital camera
Cell culture incubator (humidified, 5 % CO_2)
Biological hood with laminar flow and UV light
Pipette aid

Sterile micropipette
37 °C water bath
Centrifuge with a swing-bucket rotor, refrigerated
Hemocytometer (Burker chamber)

2.4 Basic Protocol

All procedures should be performed under sterile conditions in a biological safety cabinet using aseptic technique to prevent contamination. The procedure is standardized for HAEC (Lonza) as ECs and Matrigel (BD Biosciences) as matrix; however, other ECs or matrix extracts can also be used in substitution; in this case technique requires optimization. See also additional notes for reagent preparation.

2.4.1 Thawing and Passaging of Human Aortic Endothelial Cells (HAEC)

1. Prepare a bottle of complete EGM-2 as indicated. Note: the supplemented medium should be stored in the dark at 4 °C and should not be frozen. When stored in these conditions it is stable for 1 month.
2. Seed cryopreserved endothelial cells at 2.5×10^5 viable cells per a 25-cm^2 tissue-culture flask using 5mL EGM-2.
3. Change culture medium 24–36 h after seeding.
4. Change the medium every other day thereafter, until the culture is approximately 80 % confluent (5–6 days).
5. Using standard procedures to passage endothelial cells, split them when they are 80 % confluent. Usually, plating 5×10^5 to 1×10^6 cells in a 25-cm^2 flask works well.

The cells should be passaged at least twice after thawing before being used in the tube formation assay. However, the HAECs should not exceed passage 8–10.

2.4.2 Coating Plates with Matrigel

6. Remove Growth factor-reduced BD Matrigel from the freezer (−20 °C) and thaw in an ice bath in a refrigerator (4 °C) overnight. As other extracellular matrix preparations, Matrigel gels very easily; thus, it is important not to warm it during the thawing process and always to keep it on ice and to pre-chilled pipet-tips and plates.

7. Add 150–200 μl of Matrigel per cm^2 growth area and incubate plate for 30–60 min at 37 °C on a level surface to allow the gel to solidify. Note that 100 μl is necessary for each well in 96-well plates. It is very important to avoid bubble formation. If air bubbles get trapped in the wells, centrifuge the plate at 300xg for 10 min at 4 °C.

2.4.3 HAEC Preparation for the Assay

8. Starve cells with non-supplemented EBM-2 for 3–6 h prior to performing the assay. Then, harvest cells according to the producer instructions (Lonza).
9. Determine cell number and viability by counting cells in a Burker chamber.
10. Collect cells by centrifugation at 220xg for 5 min.
11. Aspirate supernatant and resuspend cell pellet in non-supplemented EBM-2 at a concentration of 2×10^5 cells/ml by gently pipetting up and down a few times to obtain a homogeneous single cell suspension. Be sure that cells are well mixed since cell density has an effect on tube formation (see also the point below and sect. 2.8).
12. To set the experimental points, dilute cells in non-supplemented EBM-2 in the presence or absence of angiogenesis inducers and inhibitors to be tested. Use appropriate negative and positive control samples (non-supplemented EBM-2 and complete EGM-2, respectively).

2.4.4 Starting the Assay and Analyzing Data

13. Prepare the appropriate number of HAEC cells in 1.5 ml tubes, according to the number of target cell lines to be used. Note: Per each test, one tube of HAEC will be suspended with a sufficient amount of cells to be dispensed in three wells. Thus, a triplicate will be done per each experimental point. Note that each well requires 100 μl of the HAEC cell suspension (corresponding to 2×10^4 cells/well).
14. Dispense 100 μl of the HAEC cell suspension obtained thorough mixing into the labeled wells of a 96-well plate. Be careful not to touch the surface of the gel when adding the cells to avoid injuring the gel. Incubate the plate at 37 °C, 5 % CO_2 for a period of 4–16 h, or until the desired result is achieved.
15. Visualize the cells every hour for tube formation under an inverted light microscope with 4× or 10× objective.
16. Photograph the capillary network in the wells using a digital camera attached to the inverted microscope (see also paragraph 8).
17. Analyze data using the ImageJ software (Fig. 2.1).

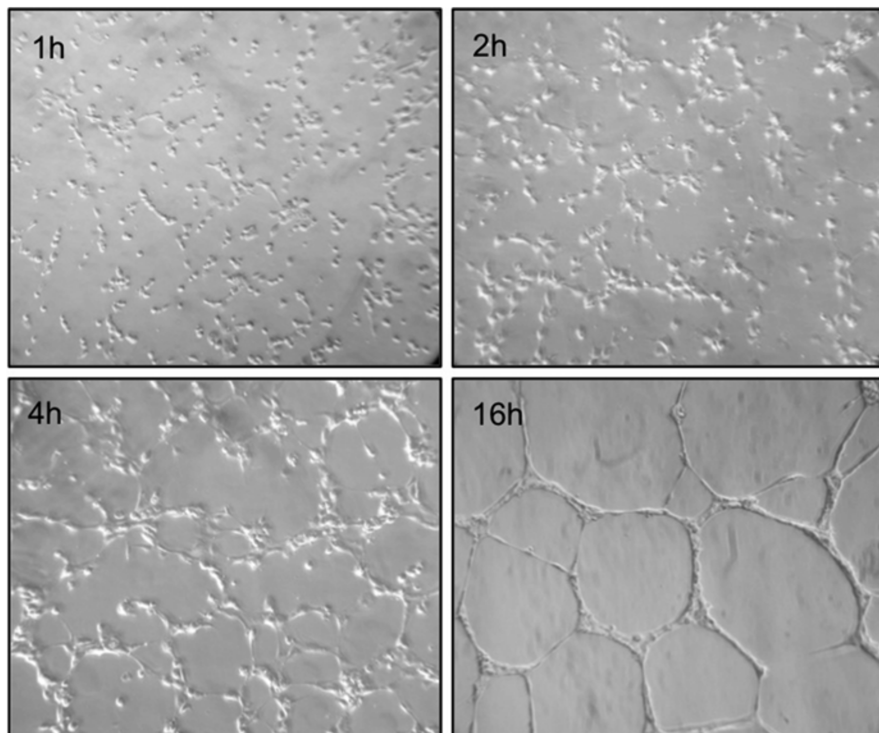

Fig. 2.1 Representative images of tube formation assay on the growth factor-reduced Matrigel by HAEC after incubation with supplemented EGM-2 for 1, 2, 4 and 16 h

2.5 Examples of Experimental Plans

This assay can be used to test in vitro a novel molecule and verify its potential as pro-angiogenic or anti-angiogenic factor. The angiogenic activity of one molecule at different concentrations or of more molecules can be easily tested simultaneously. To this purpose, cells (HAECs or alternatively HUVEC or other ECs) are diluted in non-supplemented EBM-2 medium in the presence or absence of the molecule to be assayed.

Alternatively, you can use conditioned medium from a cancer cell line to study its potential to activate angiogenesis through the release of angiogenic factors in the medium. In this setting you can test the cancer cell line under different experimental conditions. For instance, you can study the involvement of a particular gene in this process by utilizing the cancer cell line transfected to overexpress or silence that particular gene. Indeed, in our studies we have used an osteosarcoma cell line (SaOS) both un-transfected and silenced for the transcription factor YY1 [1, 2].

2.6 Sample Results

2.6.1 Data Interpretation

In this assay activated ECs form cellular networks from capillary tubes sprouting into the matrix. The formation of these networks is a dynamic process, which begins with cell migration and alignment, followed by the development and sprouting of capillary tubes, and ends with the formation of cellular networks. Although this is designed as a qualitative assay, it is also possible to quantitate the extent of the formed cellular networks. Several methods have been used for quantitation. Simple visual assessment by a blinded observer, including scoring of the tube quality or counting branch points, can provide an accurate evaluation even though often it is necessary to have an objective quantitative method, which is more rapid and easy to perform for the investigator. Many labs measure tube area, whereas others measure tube number, tube length or number of sprouts or combinations of these measurements. Some labs have adapted equipment or developed their own programs for tube formation analysis. Free software is also available, like ImageJ that can perform these measurements on the images obtained by microscopy. Similarly, website services, such as S.CORE, Angiosys and Wimasis, can also analyze data on acquired images (Fig. 2.2).

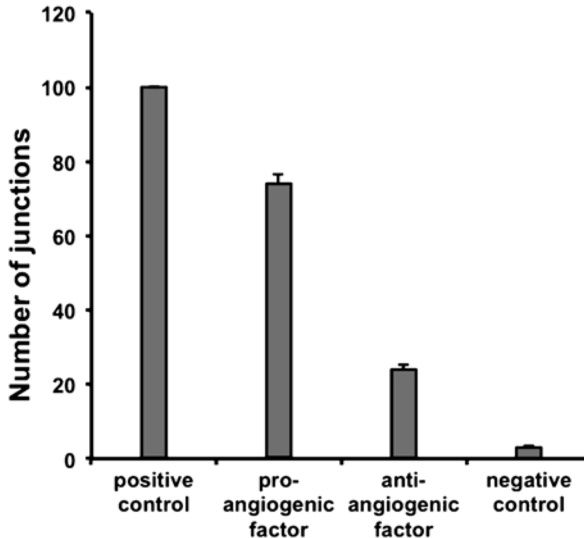

Fig. 2.2 Evaluation of the effect of pro-angiogenic and anti-angiogenic factors on HAEC tube formation. In this example of results analysis, number of junctions is represented as percent of positive control. Similar results can be plotted for tube length and for tube number

2.7 Troubleshooting

When Matrigel is too viscous, probably it is not thawed completely. Thus, keep it at 4 °C on ice until completely thawed. Another cause can be that the tube with matrigel has warmed up and started to gel. Keep the extract on ice or at 4 °C for a few hours or overnight before use.

If bubbles occur in the matrigel before or after coating, centrifuge the tube or the plate at 4 °C for 10 min at 300xg. To prevent the formation of an irregular matrigel surface, avoid touching the gel surface with the pipette tip.

Different cell density in the wells of the 96-well plate can result when cells have not been mixed properly. To avoid this problem, carefully invert the tube with cells or pipette cell suspension up and down for several times before loading the cells on the top of gelled matrigel.

When the quality of cell preparation is poor (i.e. cells from a late passage), cells may not adhere to the matrix or not to form tubes; in the last case also the number of cells used in the assay is a critical point. Indeed, if the number of cells per well is too low for the specific EC line used in the assay, no fully formed tubular structures can be observed also in the presence of pro-angiogenic factors and, hence, in the positive control. On the other hand, when the number of cells per well is too high for the specific EC line used in the assay, tube formation is observed also in the wells with a basal medium without any angiogenic factors (negative control). Thus, it would be useful to titrate the number of cells per well to establish optimal density for the cell line utilized before starting with the entire experiment. Moreover, to avoid tube formation in the negative control, be careful to properly starve cells before the assay.

2.8 Method Variations/Alternative Staining

2.8.1 Preparation of Conditioned Medium (CM) from Target Cell Line(s)

CM can be used to make the cell suspension before plating ECs on matrigel-coated plates.

1. Seed target cells with the appropriate growth medium and grow them to 30–40 % confluence (depending on the growth rate of the cell lines).
2. Replace growth medium with OPTIMEM (10 ml for a T75 tissue culture flask;) for 24–48 h.
3. Collect the CM, when cells reach 60–80 % confluence in a T75 tissue culture flask.
4. Spin at 200xg to eliminate cellular debris from the CM.
5. Make 0.5 ml aliquots of the CM and store at −80 °C if it is not immediately used after collection.

2.8.2 Fixation and Labeling with Cell Staining Solution

During all steps be careful to gently aspirate and load the following solutions in order to avoid damaging gel and the cellular network.

1. Remove the medium from the wells and rinse the wells three times with 100 μl of PBS per each well.
2. Add 100 μl of cold methanol per well and incubate the plate for 30 s–1 min. Note that longer times will result in the appearance of large precipitates of the matrix proteins that can interfere with the imaging process.
3. Rinse the wells three times with distilled water.
4. Add 100 μl of cell staining solution (a mixture of Azur A and Methylene Blue) per well and incubate the plate for 15–30 min at room temperature.
5. Rinse the wells three times with distilled water.
6. Photograph tubular networks and analyze images for quantitation.

2.9 Additional Notes

2.9.1 EGM-2 Preparation

Add all supplements and growth factors of the EGM-2 SingleQuots BulleKit to EBM-2 and store at 4 °C for up to 1 month.

2.10 Applications and Discussion

One of the most widely used in vitro assays to model the reorganization stage of angiogenesis is the tube formation assay [3]. The assay measures the ability of endothelial cells to rapidly form capillary-like structures in vitro when plated at sub-confluent densities on top of a reconstituted extracellular matrix support. Over the past several decades, researchers have typically employed this assay to determine the ability of various compounds to promote or inhibit tube formation. Anti-angiogenic factors could be useful in various diseases, including cancer, where tumors stimulate new blood vessel formation to receive oxygen and nutrients to grow.

The advantages of this assay are that it is quite easy and quick to perform, is quantifiable, and is suitable to high-throughput analysis. It also allows in vitro modeling of endothelial cell behavior, including survival, apoptosis, and the steps leading to capillary formation and invasion. A disadvantage of this assay is the great variation in the data obtained with different lots of ECs and support matrixes, thus rendering the choice of these resources crucial to get consistent and reliable data.

Recently growing evidence has shown that this assay is not limited to test vascular behavior for ECs, since certain non-endothelial cell types have also been reported to form capillary structures in vitro. Indeed, tube formation assay has also been used to test the ability of a number of tumor cells to develop a vascular phenotype, a process known as vasculogenic mimicry (VM). Tumor cell-mediated VM has been demonstrated to play a vital role in the tumor development, independent of EC-directed angiogenesis [4]. This vascular phenotype is also dependent on cell numbers plated on the Matrigel. Therefore, this assay may be useful to screen the vascular potential of a variety of cell types including vascular cells, tumor cells as well as other cells.

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Key Web-Addresses for Further Information

Web site address to download ImageJ software. http://rsbweb.nih.gov/ij/

http://www.springer.com/978-94-017-9715-3

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