Tube Formation Assay with Primary Human Umbilical Vein Endothelial Cells

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Abstract

The tube formation assay is a simple *in vitro* angiogenesis assay which is routinely used to screen the pro-angiogenic or anti-angiogenic potential of substances and compounds. It is, thus, of great utility in diseases like cancer where inhibition of angiogenesis is one of the key research areas to combat the disease. It is also applicable in limb ischemia and cardiovascular disease research where ischemia is a key component and induction of angiogenesis could be one of the means that could be tried to alleviate these diseases.

In the current study, we have optimized this assay using Lonza's Primary Human Umbilical Vein Endothelial Cells (HUVECs) and EGM™ 2 Media, and Corning's Phenol Red Free Matrigel® Matrix. Cell numbers, reagent volumes and Suramin concentrations have been optimized using two different HUVEC lots. Complete tube formation is observed within 16 hours of cell seeding and tubes can be easily visualized after staining with Calcein AM. This assay is compatible with both 48- and 96-well plate formats and is highly reproducible using our cells with EGM™ 2 Media.

Introduction

Angiogenesis is a multi-step process involving the generation of new blood vessels from pre-existing vasculature and is mediated primarily by endothelial cells. It plays a critical role not only in normal tissue repair and wound healing, but also in tumor development and cancer metastasis. Inhibition of angiogenesis is thus a key target in a number of cancers including breast, prostate, ovary, lung, colon, rectum, and brain (glioma)[1–6]. In comparison, induction of angiogenesis is required in diseases such as arteriosclerosis, myocardial infarction, limb ischemia, tissue ischemia, etc.[7,8].

Angiogenesis involves multiple steps: basement membrane disruption, endothelial cell migration, invasion, proliferation and differentiation into capillaries. One of the key steps of this process is the assembly of endothelial cells into tubes – this is known as tube formation. Tube formation can be modeled *in vitro* by plating endothelial cells onto or within extracellular matrix components like Matrigel® (isolated from Engelbreth-Holm-Swarm mouse sarcoma cells) or Type I collagen[9]. The most widely used tube formation assay involves plating HUVECs onto Matrigel® and examining the branching structures within 12–24 hours of cell plating[9,10].

Traditionally, HUVECs have been used extensively in this assay. However, the tube formation assay can also be performed with various types of endothelial cells, with endothelial progenitor cells and with transformed or immortalized endothelial cells.

While there are other assays used to measure angiogenesis *in vitro* and *in vivo*[10], the tube formation assay is simple, rapid, relatively inexpensive and accurate. Thus, it is commonly used to screen drugs or compounds for anti- or pro-angiogenic activity *in vitro*. It is therefore useful in discovering new compounds to counter diseases such as cancer or vascular ischemia, to define mechanisms and pathways involved in angiogenesis, and to identify endothelial-like cell populations or cells capable of inducing angiogenesis. Due to the high baseline levels of morphogenesis with this assay, it is more suitable for screening anti-angiogenic agents; though pro-angiogenic effects can also be measured. The effect of anti-angiogenic agents on tube formation is generally measured at later time points (12–16 hours) while the effect of pro-angiogenic factors is generally measured at earlier time points (4–8 hours).

One key advantage of using this assay is that it has various phases like cell migration and alignment, followed by the development of capillary tubes, sprouting of new capillaries, and finally the formation of the cellular networks that take place in a timed manner. Thus, varying the time point of measurement may give some information regarding the mechanism of action of an anti-angiogenic agent and the steps in the pathway it affects.

A number of inhibitors have been referenced in the literature as positive inhibitor controls for this assay. One of these inhibitors is Suramin. Suramin is a specific and competitive inhibitor of G-protein-coupled receptor (GPCR) activity and impacts multiple outputs of tubule formation, i.e. number of junctions, number of tubules and the total tubule length[11]. We have used this compound as a positive inhibitor control for the current set of studies.

Once tube formation is complete, it can be observed using an inverted microscope either in bright field, or after staining with a live cell staining dye like Calcein AM. Staining with Calcein AM enables better visualization of the tubules. Image acquisition can then be performed either manually or using an automated software.

Assessment of tube formation is basically qualitative, but some quantitation may be possible by using any one of several commercially available software systems. These systems measure different parameters

such as tubule characteristics (number of tubules, number and mean number of junctions, tubule area (%), total, mean and standard deviation of tubule length and number of independent tubules) and/or net characteristics (number of loops, mean perimeter loop and number of nets)[11]. In the current study, we have performed qualitative assessment of tube formation.

There are many references in the literature using the tube formation assay as a method for screening anti-angiogenic agents. These methods vary with regard to the extracellular matrix used, the cell type seeded, the media used for the assay, etc. In the current study, we optimized this assay using primary HUVECs and EGM™ 2 Media from Lonza to provide a well standardized, optimized and reproducible procedure for researchers to use in the screening of anti-angiogenic agents.

Materials

Cells

HUVECs (Lonza, cat. no. C2517A) of two different cell lots (lot 8F3178 and lot 0000094182) were used in this study. Cells were thawed and expanded in EGM™ 2 Growth Medium (Lonza, cat. no. CC-3162) as per instructions given in the tech sheet and were allowed to undergo at least one passage after thawing prior to use in the experiments. Cells were always used within passage 5 for best results in this assay. For donor details see Table 1.

Lot No.	Age	Sex	Race	Seeding Efficiency	Cell Viability	Doubling Time	Cell Passage	Total Populations
0000094182	Newborn	Male	H	71	76	21	1	17
8F3178	Newborn	Female	B	59	87	17	1	19

Reagents

EGM™ 2 Endothelial Growth Medium-2 BulletKit™ Kit (cat. no. CC-3162), HBSS (cat. no. BE10-547F), Trypsin/EDTA (cat. no. BE17-161E) and Trypsin neutralizing solution (TNS, cat. no. CC-5002) were from Lonza. BSA (cat. no. A-3294), Suramin (cat. no. S-2671) and DMSO were procured from Sigma. Matrigel® Basement Membrane Matrix Phenol Red Free was purchased from Corning (cat. no. 356237). Calcein AM (cat. no. C3100MP) and Pluronic acid (cat. no. P3000MP) were from Life Technologies. Tissue culture plasticware and serological pipettes were purchased from Corning.

Methods

Reagent Preparation

EGM™ 2 Endothelial Growth Medium-2 BulletKit™ Kit: The complete media was reconstituted by adding the SingleQuots™ (hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamicin/amphotericin-B) to the basal media. The complete media was kept protected from light, stored at 4°C and used within one month of reconstitution.

BSA (20 mg/mL): The stock solution was prepared by dissolving 0.5 gm BSA in 25 mL HBSS. It was stored at 4°C and used within 15 days of preparation.

Calcein AM stock (1 mg/mL): The stock solution was prepared by adding 50 µL DMSO to the vial containing the 50 µg solid. It was stored in aliquots at -20°C and kept protected from light.

Calcein AM staining dye: The following components were mixed to make the dye: 20 µL Calcein AM stock (1 mg/mL stock), 20 µL Pluronic acid and 2 mL BSA stock solution (20 mg/mL stock). The Calcein AM staining dye was prepared just before use and kept protected from light. The Calcein AM dye was diluted in HBSS and not in media since culture medium results in the auto-hydrolysis of the label giving high fluorescence background.

Suramin (300 µM stock): The stock solution was prepared by dissolving 0.42 gms of Suramin (Mol. wt. - 1429.17) in 1 mL of HBSS. It was prepared just before use and kept protected from light.

Thawing the Matrigel®

Matrigel® with a minimum protein concentration of 10 mg/mL is required to obtain optimal results in this assay[12, 13]. Thus, we used Corning Matrigel® for the current assay. The Phenol Red Free variant of Matrigel® was used in our assays. The Phenol Red Free variant was selected since phenol red has estrogenic activity.

The Matrigel® was thawed by placing it overnight on ice and keeping the ice bucket in the refrigerator at 4°C, since Matrigel® may gel at the slightly elevated temperatures in a refrigerator. Immediately after thawing, the Matrigel® was aliquoted out into smaller volumes and stored at -20°C. Since Matrigel® gels rapidly at 22°C to 35°C, pre-cooled pipettes were used and Matrigel® was kept on ice during the handling process.

Coating Culture Plates with Matrigel®

The culture plates and tips were pre-cooled by placing in the refrigerator overnight and also kept on ice during the coating process. The required number of wells was coated with Matrigel® using pre-cooled pipette tips; 150 µL per well was used for coating 48-well culture plates and 75 µL per well was used for coating 96-well culture plates. While coating the wells with Matrigel®, care was taken to avoid introducing bubbles in the well. However, if air bubbles did get trapped in the wells, the plate was centrifuged at 300 g for 10 minutes in a pre-cooled centrifuge at 4°C. Plates were coated just before the experiment. The plate was left undisturbed in the biosafety cabinet for 10 minutes at room temperature. After 10 minutes, the Matrigel®-coated plate was gently shifted to a CO_2 incubator at 37°C. It was left in the incubator for 30 minutes to allow for gelation of the Matrigel®. During this incubation process, the cell cultures were trypsinized and made ready for the assay.

Cell Growth and Trypsinization

Cells were grown to 70–80% confluence in complete EGM™ 2 Media. Once they reached confluence, they were detached from the cell surface by washing once with HBSS, trypsinizing and resuspending in TNS as per the recommended protocol. TNS was used to stop the action of Trypsin-EDTA since EGM™ 2 Media has a low concentration of FBS (2%). Cells were spun down by centrifugation at 1,200 rpm for 10 minutes at room temperature. The supernatant was discarded and cells were resuspended in complete EGM™ 2 Media. The cell viability was determined using Trypan blue. Cells were used for the assay only if the cell viability was greater than 80%. Cells were used in early passages (up to passage 4) and they were passaged at least once after being removed from liquid nitrogen before being used for this assay.

Experimental Set Up

Cells were diluted in complete EGM™ 2 Media at a concentration of 0.4 x 10^6 cells/mL, i.e., 100,000 cells in 250 µL complete media. Requisite dilutions of the above cell suspension were then made in Eppendorf tubes. Angiogenesis inhibitors were also added to the Eppendorf tubes, along with the cells, such that 250 µL of final cell suspension contained the required number of cells for the assay and the requisite concentrations of the angiogenesis inhibitor. The above cell suspension was then added on to the Matrigel®-coated wells of a 48-well (250 µL per well) or a 96-well (125 µL per well) plate. Suramin was added in parallel as a positive inhibitor control in each assay.

In the initial set of experiments, a cell-seeding optimization experiment was performed where a range of cells from 25,000–75,000 cells per well were added in a 48-well format and 12,500–37,500 cells per well were added in a 96-well plate format.

Once the cell number was optimized (50,000 cells per well in a 48-well plate format or 25,000 cells in a 96-well plate format) for optimal tube formation (Figures 1 and 2), the optimized cell number was used for the subsequent Suramin experiments.

Staining with Calcein AM

Once the incubation was over, the wells were washed gently with HBSS, taking care not to disrupt the tubules. Calcein AM staining dye was added to the wells (250 µL per well in a 48-well plate or 125 µL per well in a 96-well plate) and the cells were incubated in the dark for 30 minutes at 37°C in a CO_2 incubator. The extra dye was removed carefully by giving two washes with HBSS, taking care not to disturb the tubules. The tubules were observed under an epifluorescence microscope using a standard FITC filter.

Results and Discussion

The current assay has been standardized and optimized using Lonza's HUVEC primary cells, EGM™ 2 Media and Corning's Phenol Red Free Matrigel® Matrix (extracellular matrix gel prepared from Engelbreth-Holm-Swarm tumor cells). The assay results have been shown for only one cell lot (lot no. 8F3178) since both lots gave similar results.

It is known, based on the literature, that tube formation starts within 4 hours after plating the cells, complete tube formation takes about 12–16 hours, tubules start getting disrupted after 16–18 hours and the cells begin to undergo apoptosis after 24 hours using a different extracellular matrix. We repeated this assay using our HUVECs and EGM™ 2 Media at some of the above time points and found that tubules start getting disrupted after 16 hours of cell plating, and they appear to detach from the cell surface at 24 hours after cell plating (data not shown). As a result, we chose 16 hours as the ideal time point for the assessment of tube formation.

Figures 1 and 2 depict bright field and fluorescence images of HUVECs (lot no. 8F3178) plated at varying cell densities of Matrigel® in EGM™ 2 Media and their tube formation at 16 hours after cell plating in a 48-well plate and a 96-well plate format respectively. These cell optimization experiments demonstrated that the number of cells plated is critical: too few cells yield incomplete tubes (i.e., 25,000 cells per well using a 48-well plate format), while too many yield large areas of cell clusters or monolayers (i.e., 75,000 cells per well using a 48-well plate format). The optimum cell number for this assay was found to be 50,000 cells per well for a 48-well plate format and 25,000 cells per well for a 96-well plate format. Similar results were obtained with lot no. 0000094182 (data not shown), demonstrating the reproducibility of the assay across cell lots and across 48- and 96-well plate formats.

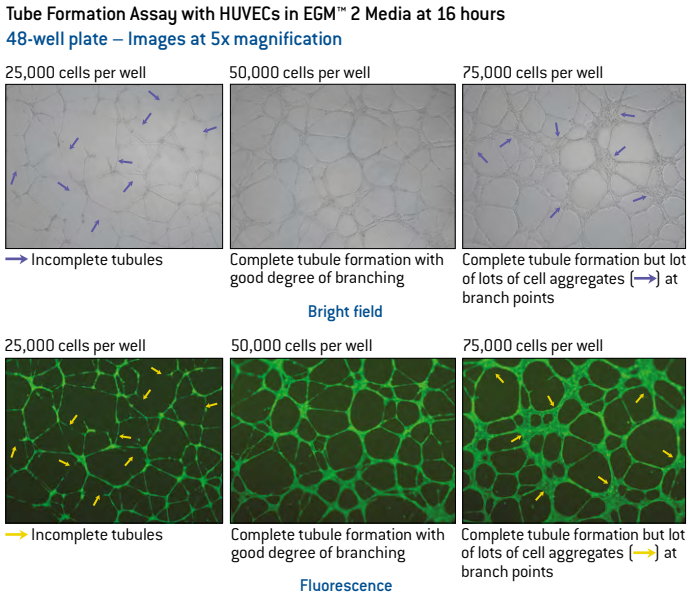

Figure 1. Figure 1 depicts cell number optimization for the tube formation assay using HUVECs (lot no. 8F3178) seeded on Matrigel® in EGM™ 2 Media at a 16-hour assay point. This assay was conducted in a 48-well plate format. Both bright field and fluorescence images have been depicted here and indicate that 50,000 cells per well is the optimal cell seeding concentration for this plate format.

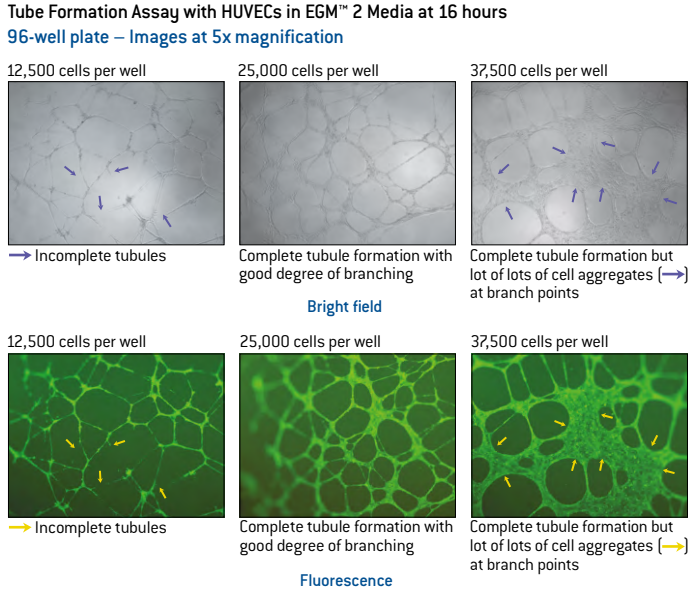

Figure 2. Figure 2 depicts cell number optimization for the tube formation assay using HUVECs (lot no. 8F3178) seeded on Matrigel® in EGM™ 2 Media at a 16-hour assay point. This assay was conducted in a 96-well plate format. Both bright field and fluorescence images have been depicted here and indicate that 25,000 cells per well is the optimal cell seeding concentration for this plate format.

Suramin, when used at a concentration of 7.5–60 µM, was found to inhibit the tubule branches in lot no. 8F3178 cells in a dose-dependent manner (Figure 3), in concurrence with the literature. Suramin effect was tested again in lot no. 0000094182 cells at 15 µM and 30 µM concentrations (data

not shown), and it appeared that Suramin effect was lot-specific and that the degree of inhibition of tube formation across tested lots appeared to vary slightly.

Effect of Suramin on HUVEC Tube Formation in EGM™ 2 Media at 16 hours
Suramin

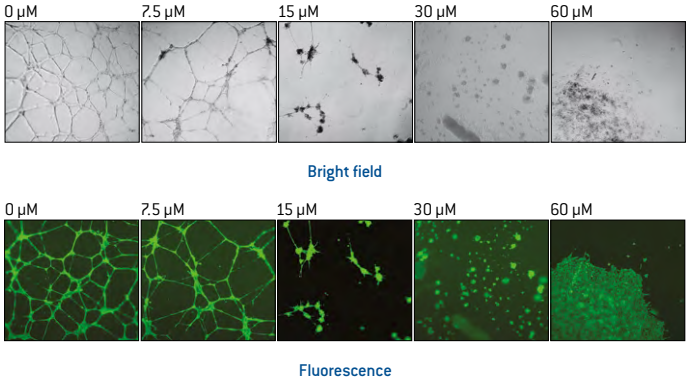

Figure 3. Figure 3 depicts the effect of the inhibitor Suramin on tube formation assay of HUVECs (lot no. 8F3178) seeded on Matrigel® in EGM™ 2 Media at a 16-hour assay point. This assay was conducted in both 48- and 96-well plate formats and both gave similar results. Only the 48-well plate data has been presented here. Both bright field and fluorescence images are depicted here and they indicate that Suramin, at a concentration of 15–30 µM, can be ably used to demonstrate the inhibitory effect of Suramin on the tube formation assay.

Conclusion

In conclusion, we found that the current assay using Lonza's HUVEC Primary Cells and EGM™ 2 Media offers a well standardized, optimized and reproducible platform for researchers wishing to use the tube formation assay for screening anti-angiogenic agents using either 48-well or 96-well formats.

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