An Automated Endothelial Cell Tube Formation Assay System for the Screening of Angiogenesis Stimulatory and Inhibitory Molecules

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Abstract

A tractable cellular model to validate drug targets and for screening efficacious drug candidates is highly desirable. Endothelial cell differentiation into capillary-like structures on BD Matrigel™ Matrix is widely used to study effects of molecules that may modulate angiogenesis. However, this assay is mostly non-quantitative and it is difficult to assess the effect of angiogenesis activators. BD Biosciences has overcome these limitations. We present an automated and rapid *in vitro* endothelial cell tube formation assay that allows the screening of potential angiogenesis stimulators and inhibitors. Modified growth factor reduced (GFR) BD Matrigel Matrix, on which tube formation in the absence of exogenous angiogenesis activators is greatly reduced comparing to that on standard GFR BD Matrigel Matrix, was placed in 96-well plates. HUVECs were plated in the well of polymerized BD Matrigel Matrix in the presence or absence of angiogenesis stimulators and labeled with Calcein AM at the end of an assay. Images were acquired and analyzed by automated algorithms using MetaMorph® Software. Utilizing the Angiogenesis application module, multiple parameters measuring the extent of tube formation, such as tube length, tube area, tube thickness, and number of branch points, were used for quantification. We measured the dose response of bFGF and Spinosine-1 Phosphate (S1P) induced HUVEC tube formation using tube length, tube area and number of branch points. EC_{50} values obtained using the three parameters were nearly identical. IC_{50} values of Suramin against bFGF and S1P induced tube formation were found to be very close using the same three parameters. Similar IC_{50} values were also found using manual measurements. Furthermore, we tested the effects of various signaling pathway inhibitors and showed they interfere with tube formation in a slightly different manner. This rapid and reliable assay system would facilitate pre-clinical screening of potential angiogenesis stimulatory and inhibitory molecules and drug action mechanism studies.

Introduction

Endothelial cell tube formation on BD Matrigel™ Matrix has been widely used in angiogenesis research and drug discovery. BD Biosciences previously developed an endothelial cell tube formation assay system based on regular BD Matrigel Matrix, which is very useful for studying angiogenesis inhibitors. In order to develop a tube formation system for studying angiogenesis stimulators, we modified standard growth factor reduced (GFR) BD Matrigel Matrix in which background tube formation in the absence of exogenous activator is greatly reduced. Automation allows image acquisition of a 96-well plate to be completed in approximately 10 minutes on a Gen-1 Cell-based screening system. Furthermore, utilization of Angiogenesis application module within the MetaMorph® Software enabled us to obtain data measuring multiple parameters of endothelial cell tube formation.

Materials and Methods

Cell culture and reagents – Human umbilical cord endothelial cells (HUVEC) from Cascade Biologics were cultured in EGM2-MV medium from Cambrex in BD Falcon™ tissue-culture treated flasks. Hank's Balance Salt Solution (HBSS) was obtained from Life Technologies. Human recombinant bFGF was obtained from BD Biosciences. Sphigosine-1 Phosphate (S1P), Ly294002, GF109203x, U0126, and Suramin were obtained from CALBIOCHEM. Calcein AM was purchased from Molecular Probes.

Preparation of endothelial cell tube formation assay system – 50 µl of modified GFR BD Matrigel Matrix (reconstituted basement membrane, BD Biosciences) at optimal concentration was added to BD Falcon Black/Clear 96-well microplates. A mat cover was used to seal each plate before replacing the lid. The plates were frozen and stored at -20°C before use.

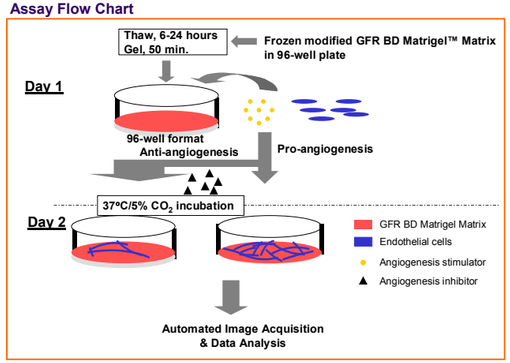
Endothelial cell tube formation assay – BD Matrigel Matrix plates were thawed at 4°C for a period of 6-24 hours. They were allowed to polymerize with mat cover at 37°C for 45-55 minutes immediately before assay setup. For activator studies, HUVEC (P3-P5) were harvested at approximately 80% cell confluence. 50 µl cells at 4 x 10^5/ml, (i.e., 2,000 cells/well), in 0.1% FBS/EBM-2 medium alone or supplemented with specific angiogenesis activators were seeded onto pre-solidified modified GFR BD Matrigel Matrix. The assembled assays were allowed to incubate in 37°C incubator with 5% CO_2 for 16-18 hours. In the inhibitor studies, indicated compounds were included in assays in addition to specified stimulators. Compounds were dissolved in appropriate solvents according to manufacturers' instruction.

Automated image acquisition – At the end of each assay, assay medium was removed and plates were washed once with HBSS. 50 µl Calcein AM at 8 µg/ml in HBSS was added to each well, and plates were incubated in 37°C incubator for 40-50 minutes. Images were acquired with a 2X objective lens using Gen-1 Cell-based Screening System (Universal Imaging Corporation).

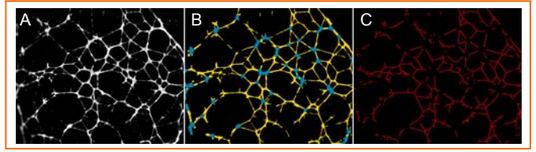
Automated image analysis – The Discovery-1™ Angiogenesis application module was used to analyze the images. Approximate minimum and maximum tube widths were entered as well as a minimum image contrast level. The Angiogenesis application module then used Adaptive Background Correction™ to calculate tubule measurements, branch points, and other parameters, and was logged to Excel for further analysis. Result images demonstrating the segmentation of tubules and locations of nodes were produced for manual inspection of the data. Manual measurements of tubule length were made using the Region of Interest tools within the MetaMorph® Software.

EC_{50} and IC_{50} determination – Excel data was imported and analyzed using BD Gentest™ MPM/ADMET Software Program (BD Biosciences Discovery Labware).

Assay Flow Chart

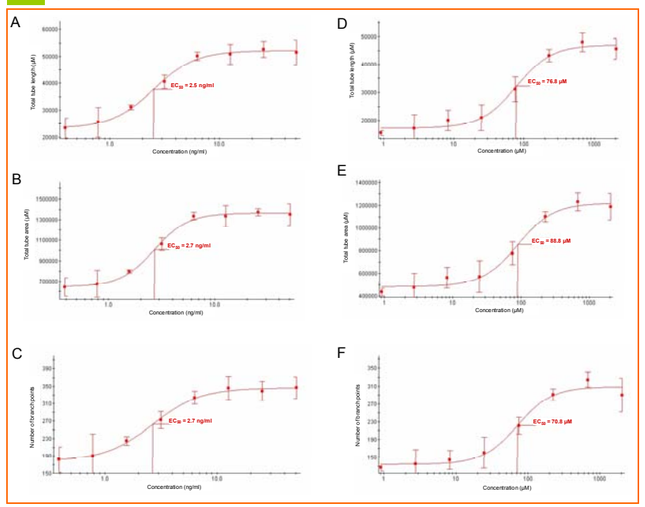


1 Segmentation and identification of tubes and nodes



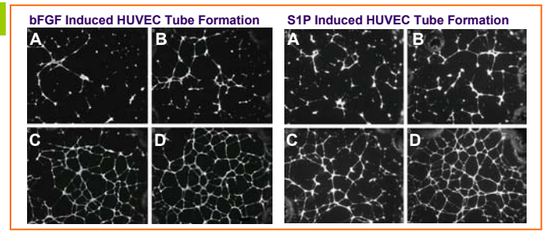
Segmentation and identification of tubes and nodes. (**A**) Original raw image. (**B**) Same images with tubes outlined yellow and nodes in blue. (**C**) Dilated skeleton of tubes.

2 Dose response of HUVEC tube formation to bFGF and S1P



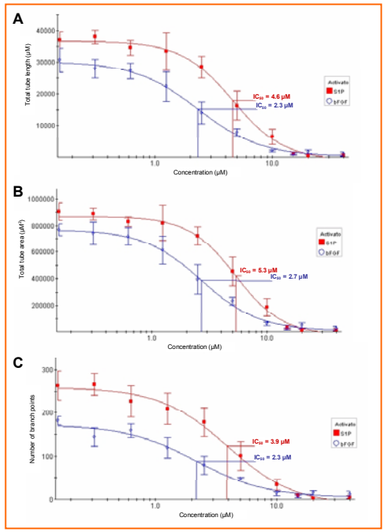
Dose response to bFGF (**A,B,C**) and S1P (**D,E,F**). Three parameters were used in determining EC_{50} value for bFGF and S1P respectively. The EC_{50} values as indicated for each activator are similar when three parameters were measured. (**A** and **D**) tube length; (**B** and **E**), tube area; and (**C** and **F**), number of branch points. Each data point represents the mean ±S.D. (n=4).

3



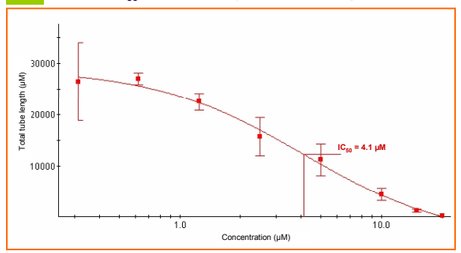
Representative images of HUVEC tube formation in response to bFGF (*Left Panel*): (**A**) 0 ng/ml; (**B**) .78 ng/ml; (**C**) 3.1 ng/ml; (**D**) 12.5 ng/ml. HUVEC tube formation in response to S1P (*Right Panel*): (**A**) 0 nM; (**B**) 8 nM; (**C**) 74 nM; (**D**) 667 nM.

4 Dose response of Suramin inhibiting bFGF and S1P induced HUVEC tube formation



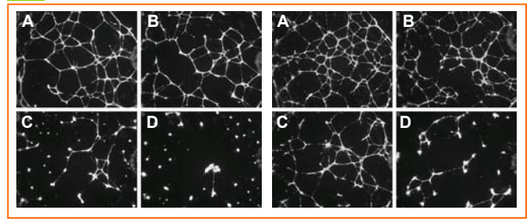
Dose response of Suramin in inhibiting bFGF and S1P mediated tube formation. Three parameters, (**A**) tube length, (**B**) tube area, and (**C**) number of branch points were used in determining IC_{50} values. The Suramin IC_{50} value of each signaling pathway is very close as determined using all three parameters. Each data point represents the mean ±S.D. (n=4).

5 Suramin IC_{50} determined by manual scoring



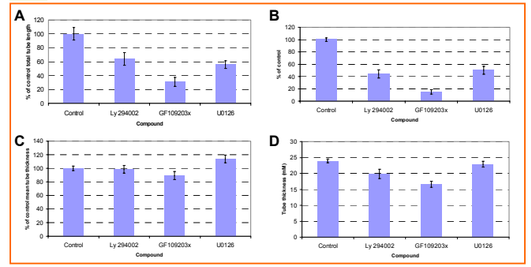
Manual scoring of tube length dose response of Suramin in inhibiting bFGF mediated HUVEC tube formation. The same set of images used for automated analysis were used here. The Suramin IC_{50} value as indicated is very close to that obtained by automated scoring in ***Figure 4***. Each data point represents the mean ±S.D. (n=3).

6 Suramin inhibits bFGF and S1P induced HUVEC tube formation



Representative images of Suramin inhibition of HUVEC tube formation induced by bFGF (*Left Panel*) and S1P (*Right Panel*) respectively. (**A**) 0 µM; (**B**) 0.62 µM; (**C**) 5 µM; (**D**) 10 µM

7 Effects of inhibitors of distinct signaling pathways on bFGF and S1P induced HUVEC tube formation



Inhibition of bFGF (**A** and **C**) and S1P (**B** and **D**) induced tube formation by PI3 kinase, PKC and MEK1 inhibitors Ly294002, GF109203 and U0126 respectively as measured by tube length (**A** and **B**) and tube width (**C** and **D**). The concentration of all three compounds is 10 µM. Each bar represent the mean ±S.D. (n=7).

Summary

1. Modified GFR BD Matrigel Matrix based Endothelial Cell Tube Modulation Assay System allows assessment of the effects of both angiogenesis stimulators and inhibitors.
2. Multiple data sets generated using Angiogenesis application module showed similar IC_{50} and EC_{50} values for individual angiogenesis activators and inhibitors, and the data are consistent with literature.
3. Manual and automated scoring of same images produced similar results.
4. The rapid assay in combination with automated image acquisition and analysis should be applicable to screening both angiogenesis activators and inhibitors.