ORIGINAL ARTICLE

Comparison of invasion by human microvascular endothelial cell lines in response to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in a three-dimensional (3D) cell culture system

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Abstract

Background: Immortalized human endothelial cells are widely used as *in vitro* models for debilitating conditions such as cancer, cardiovascular and ocular diseases. Human microvascular endothelial cell (HMEC-1) is immortalized via stable transfection with a gene encoding SV40 large antigen whilst telomerase-immortalized human microvascular endothelial (TIME) cells is immortalized by engineering the human telomerase catalytic protein (hTERT) into primary microvascular endothelial cells. Here, we established a three-dimensional (3D) spheroid invasion assay with HMEC-1 and TIME and compared the difference in their ability to invade through the collagen matrix in response to exogenous growth factors, namely vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). *Methods:* TIME and HMEC-1 spheroids were embedded in a collagen matrix. The spheroids were stimulated with exogenous growth factors, namely VEGF (50ng/mL) and bFGF (200ng/mL). Twelve points of invasion length from a spheroid was measured using image analysis software, Image J. Three independent experiments were conducted and data was analysis by GraphPad Instat software, version 3.05. *Results:* TIME spheroid invasion was 16.5 fold higher with exogenous VEGF (50ng/mL) and bFGF (200ng/mL) treatment as compared to those cultured in complete growth medium only. In contrast, no significant difference was observed between HMEC-1 spheroids stimulated with and without exogenous growth factors, VEGF and bFGF. *Conclusions:* This is the first report on the establishment of a 3D-spheroid invasion assay with TIME cells. The requirement of VEGF and bFGF for TIME spheroids invasion is a novel finding. In addition, this assay offers an advantage over HMEC-1 for testing novel angiogenic agents since it is not affected by endogenously secreted growth factors.

Keywords: endothelial cell invasion, TIME, HMEC-1, VEGF, bFGF

INTRODUCTION

Angiogenesis is a process in which new blood vessels are formed and is essential in growth and development as well as in wound repair. Abnormal blood vessel growth is associated with many debilitating conditions such as cancer, ocular diseases, diabetic wounds that do not heal, and cardiovascular diseases. The essential role of angiogenesis in tumour growth was first hypothesized in 1971 by Folkman.[1] Tumour angiogenesis is characterized by a number of cellular events including endothelial cell migration, invasion and differentiation into capillaries. Invasion is a process in which cell movement through a 3D matrix, accompanied by a restructuring of the 3D environment. Spheroid invasion assay, which involves movement of cells through a 3D matrix, closely mimics invasion *in vivo* because invasion occurs from cell clusters rather than from a single cell layer.[2] The advantages of this assay are its simplicity, reproducibility, and similarity to physiological tissues as compared to other methods.[3] Recently, tumour spheroids have been commonly used as models to test efficacy of drug delivery,[4] tumour cell cytotoxicity,[5] anti-cancer drug therapeutic

efficiency[6] and effects of drug on tumour growth and invasion.[7]

Until relatively recently, the human umbilical vein endothelial cell (HUVEC) has been the most commonly used for *in vitro* studies in angiogenesis. However, HUVEC is not immortalized and dies off after a number of passages and thus, new alternatives are required. Immortalized endothelial cells are now widely used as *in vitro* model systems for angiogenesis studies. These immortalized cell lines are more stable and show better characteristics.[8] The most commonly used are human microvascular endothelial cell line (HMEC-1). HMEC-1 is immortalized via stable transfection with a plasmid containing the Simian Virus (SV) 40 large antigen gene and has been used for a wide range of applications in endothelial cell research and drug development.[9] Another immortalized endothelial cell line is the telomerase-immortalized human neonatal dermal microvascular endothelial cell line, designated as TIME. These immortalized cell lines provide a convenient model for *in vitro* studies.

HMEC-1 has been shown to produce angiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which can induce HMEC-1 spheroid invasion.[10] However, the invasiveness property of TIME spheroids in collagen embedding 3D model system is not known. The objectives of this study are to establish a 3D-spheroid invasion assay with HMEC-1 and TIME and to compare their differences in spheroid invasion in response to exogenous angiogenic factors, namely, VEGF and bFGF. The results obtained will provide an insight into the appropriate choice of a 3D-spheroid invasion assay for screening of angiogenic and anti-angiogenic agents as well as for elucidating the mechanisms underlying spheroid invasion when co-cultured with tumour cells in the future.

MATERIALS AND METHODS

Cell culture and medium
TIME was purchased from American Type Culture Collection (ATCC, USA) and cultured in Lonza EGM™-2-MV BulletKit (Lonza, Walkersville, MD, USA), which is the basal medium and Single Quots Kit consisting of 5% (v/v) fetal bovine serum (FBS), hEGF (human recombinant Epidermal Growth Factor), hydrocortisone, hFGF-B (human Fibroblast Growth Factor Basic with heparin), VEGF, R3-IGF-1 (Human Recombinant Insulin-like

Growth Factor), Ascorbic Acid, and additional supplemented with 12.5 µg/mL blasticidine S (Lonza, USA) at 37 °C and 5% CO_2.

HMEC-1 which was generously provided by the Centre for Disease Control (CDC, USA) was cultured in endothelial basal medium MCDB 131 (Gibco, USA) supplemented with a final concentration of 1 µg/mL hydrocortisone (Sigma-Aldrich, USA), 10 ng/mL EGF (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, USA), and 10% (v/v) FBS at 37°C, 5% CO_2. The percentage of cell confluence was observed by viewing with an inverted phase contrast microscope. Once the cells have reached 80% to 90% confluency, sub-culturing was performed.

Three-Dimensional (3D) Spheroid Invasion Assay
Preparation of 4% agarose
A stock solution of 4% agarose (w/v) was prepared with 0.04 g of agarose powder (Sigma-Aldrich, St. Louis, MO) and 1 ml of distilled water followed by autoclaving at 120 °C. The dissolved agarose was cooled down at room temperature before transfer to 4°C.

Coating of 96-well plates with 2% agarose
Agarose (4%) from 4°C was heated in a heating block at 80°C for 10 minutes and then 50°C for another 10 minutes. Agarose was further diluted in equal volume of Dulbecco's Modified Eagle Medium (DMEM/F12) (GIBCO®, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) of FBS. The 96-well plates were rinsed with 50 µL of RPMI (GIBCO®, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS before coating with 50 µL of 2% agarose. The 96-well plates coated with agarose were stored at 4°C for 1 hour to solidify the agarose.

Three-dimensional (3D) spheroid invasion assay
The 3D-spheroid invasion assay was performed in EGM™-2-MV BulletKit consisting 5% (v/v) FBS with additional supplemented of 12.5 µg/mL blasticidine S for TIME and MCDB-131 with additional supplemented of 1 µg/mL hydrocortisone, 10 ng/mL EGF, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (v/v) FBS for HMEC-1. These were seeded into 96-well plates pre-coated with 2% agarose at a density of 10000 cells per 100 µL and centrifuged at 278 g. Once the cells were aggregated, the plate was placed in an incubator overnight at 37°C, 5%

CO_2 to promote spheroid formation. After 2 days, spheroids were harvested for embedding.

Spheroid embedding

A stock solution of 8.5 mg/mL of rat tail collagen type-I (BD Biosciences, UK) was stored at 4°C. The pH value of collagen was neutralized by mixing with RPMI (10X). In 1 mL of collagen mixture preparation, collagen mixture containing 235.2 μL of collagen type-I (8.5 mg/mL), 125 μL of methylcellulose (2%; Sigma-Aldrich, St. Louis, MO), 100μL of RPMI (10X), and 539.8 μL of distilled water. The mixture was vortexed several times to ensure complete mixing and kept on ice before being used for embedding. Each spheroid was transferred carefully into a 0.5 mL eppendorf tube with a pipette. The spheroid was re-suspended in 50 μL of collagen mixture immediately. Then, the collagen mixture containing the spheroid was dispensed slowly into 2% agarose-coated 96-well plates and incubated at 37°C, 5% CO_2 for 1 hour. After solidification, 100 μL of growth factor supplemented medium with or without exogenous angiogenic growth factors, 50 ng/mL of VEGF (R&D Systems, Minneapolis, MN, USA) and 200 ng/mL of bFGF (GIBCO®, Invitrogen, Carlsbad, CA, USA) were added to the wells. The spheroid cells were allowed to invade and photographs were taken every 12 hours.

Quantification of Invasion

The endothelial cells invading through the collagen matrix of the spheroids were visualized and imaged under Olympus IX51 inverted microscope (Olympus Corporation, Tokyo, Japan) and images were captured at 100X magnification. In each spheroid, twelve points of invasion length from spheroid were measured. The invasion length from a spheroid was measured using image analysis software, Image J (National Institutes of Health, USA). Three independent experiments were conducted. Group mean values and standard deviations were calculated. Data was analysis by GraphPad Instat software for Windows version 3.05 (GraphPad Software, San Diego, CA). Parametric data was analysis by unpaired t-test. All data were presented as mean ± standard deviation (SD). P < 0.05 is considered statistically significant.
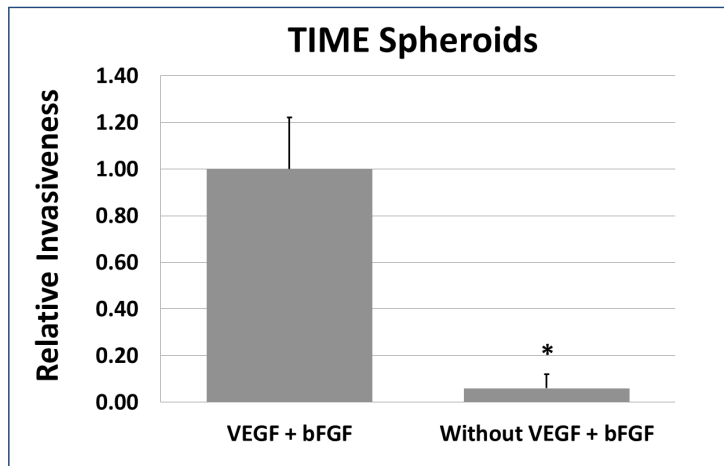
RESULTS

TIME spheroids

Figure 1 shows the morphological changes of TIME spheroids at 0 hour, 12 hours and 24 hours. At 12 hours, no invasion was observed on TIME spheroids. However, at 24 hours cells in the TIME spheroids were observed to have invaded into the collagen matrix. Figure 2 shows the quantitative analysis of invasion for TIME at 24 hours. A significant difference between
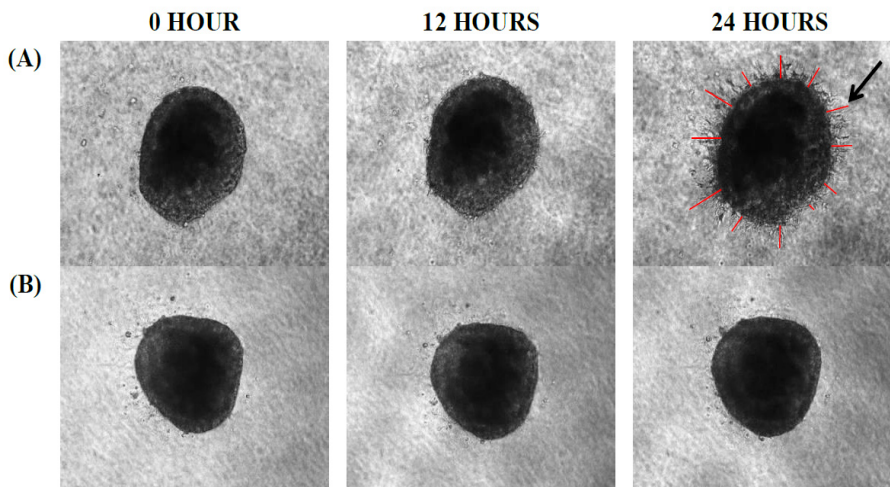
FIG. 1: Effect of exogenous angiogenic growth factors, VEGF and bFGF on invasion by TIME spheroids. TIME spheroids were embedded in a collagen matrix in the presence of complete media (EGM™-2-MV + 5% FBS) supplemented with (A) or without (B) VEGF (50 ng/mL) and bFGF (200 ng/mL). The invading cells were visualized and imaged under Olympus IX51 inverted microscope and images were captured at 100X magnification at 0, 12, 24 hours of treatment. Arrow shows invasion length projected from a spheroid.
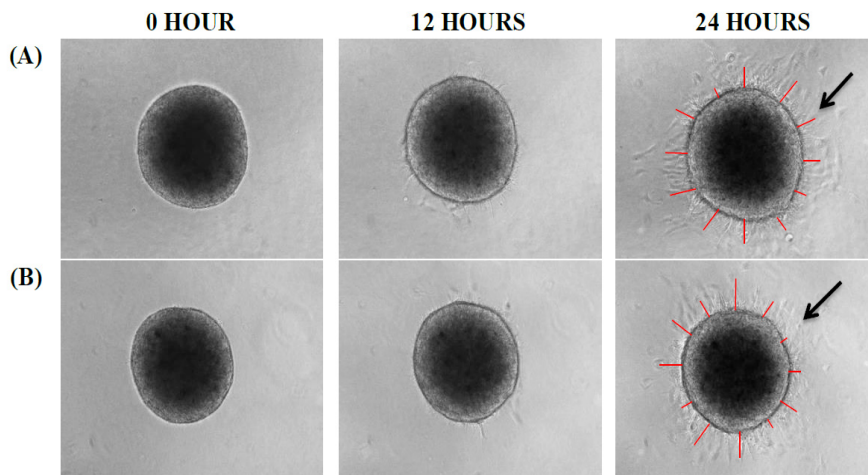
TIME Spheroids

FIG. 2: Quantitative analysis of invasion by TIME spheroids at 24 hours. The ratio of invasion length of TIME spheroid with and without addition of exogenous angiogenic stimulus, VEGF and bFGF was calculated. Quantified results are expressed relative to spheroids without addition of VEGF (50ng/mL) and bFGF (200ng/mL) are presented in the bar chart. Each column represents mean ± SD of three independent experiments compared with control at 24 hours ($n = 3$, *$P < 0.05$)

TIME spheroids with and without exogenous VEGF and bFGF treatment was found (P <0.05). The invasiveness of TIME spheroids cultured in EGM™-2-MV with addition of VEGF (50 ng/mL) and bFGF (200 ng/mL) was 16.5 fold higher as compared to TIME spheroids cultured in complete growth medium only. Our result suggests that addition of exogenous angiogenic factors, VEGF and bFGF, enhanced invasion of TIME spheroids in 3D culture suggesting that

VEGF and bFGF play important roles for the invasiveness of TIME spheroids.

HMEC-1 spheroids

Figure 3 shows the morphological changes of HMEC-1 spheroids at 0 hour, 12 hours and 24 hours. At 12 hours, cells in HMEC-1 spheroids started to invade into collagen. At 24 hours, more cells in HMEC-1 spheroids invaded into collagen. Figure 4 shows the quantitative analysis of

FIG. 3: Effect of exogenous angiogenic growth factors, VEGF and bFGF on invasion by HMEC-1 spheroids. HMEC-1 spheroids were embedded in a collagen matrix in the presence of complete media (MCDB131 + 10% FBS) supplemented with (A) or without (B) VEGF (50 ng/mL) and bFGF (200 ng/mL). The invading cells were visualized and imaged under Olympus IX51 inverted microscope and images were captured at 100X magnification at 0, 12, 24 hours of treatment. Arrow shows invasion length projected from a spheroid.
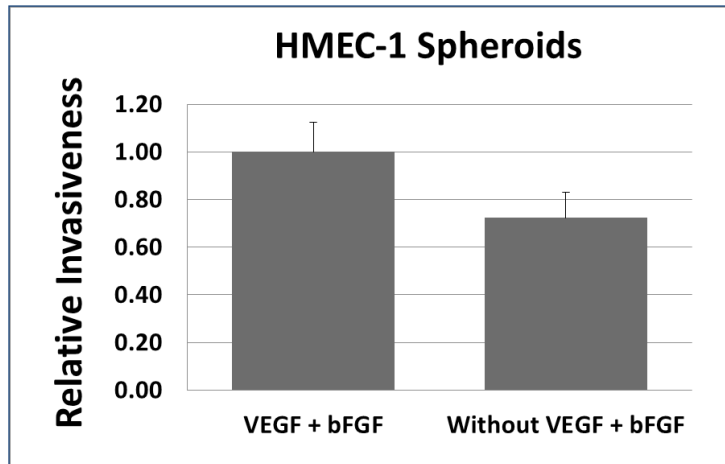
FIG. 4: Quantitative analysis of invasion by HMEC-1 spheroids at 24 hours. The ratio of invasion length of HMEC-1 spheroid with and without addition of exogenous angiogenic stimulus, VEGF and bFGF to spheroids with VEGF and bFGF was calculated. Quantified results are expressed relative to spheroid without addition of VEGF (50ng/mL) and bFGF (200ng/mL) are presented in the bar chart. Each column represents mean ± SD of three independent experiments compared with control at 24 hours ($n = 3$, P=0.062)

invasion for HMEC-1 at 24 hours. HMEC-1 spheroids stimulation with exogenous growth factors VEGF (50 ng/mL) and bFGF (200 ng/mL) was 1.4 fold higher as compared to HMEC-1 spheroids without stimulation. This result is not significantly different suggesting that invasion of HMEC-1 spheroid occurred without requiring exogenous growth factors VEGF and bFGF.

DISCUSSION

Basic fibroblast growth factor was first covered as angiogenic growth factor in superfamily of FGF.[11] Synergism of bFGF and VEGF on endothelial cell function had been reported in several studies.[12,13] Angiogenic activity stimulated with both VEGF and bFGF were found occurs faster in a preclinical study of microvascular endothelial cell model as compared to single alone.[12] Moreover, angiogenesis of pancreatic beta cell carcinogenesis in transgenic mouse model was reported synergistically inhibited by blocking VEGF and bFGF receptors.[14] Hence, both VEGF and bFGF were used to stimulate invasion of TIME and HMEC-1 spheroids in our 3D culture system.

Immortalized microvascular endothelial cells, namely TIME and HMEC-1 were chosen for this study because immortalized endothelial cells have an extended lifespan, whereas HUVECs have a limited lifespan.[8] HUVECs stop proliferating and eventually die after an average

of 10 serial passages.[15] In contrast, TIME cells are able to proliferate for at least 200 population doublings,[16] and for HMEC-1, it was reported that no senescence was shown even when the cells passaged up to 95 times.[9] In addition, HUVECs display characteristics that vary from batch to batch due to their different donor origin.[8] Therefore, this primary endothelial cell, HUVEC is not suitable for long-term *in vitro* experiments unlike HMEC-1 which retains the phenotypical, morphological and functional characteristics similar to the primary cells such as expression of endothelial cell-surface molecule CD31[9,17] and CD36, expression of receptors for low-density lipoprotein and tubule formation on matrigel.[8,9] Similarly, TIME also display phenotypes which are characteristic of its origin such as surface marker protein, CD31/PECAM-1 and v 3-integrin, retain tubule formation ability when plated on matrigel and also express receptors for low-density lipoprotein.[16]

Comparison of the results between TIME and HMEC-1 spheroid invasion showed that TIME spheroid invasion required additional exogenous stimulus, VEGF and bFGF whereas in the case of HMEC-1, spheroid invasion occurred without VEGF and bFGF. We propose that this could be due to the endogenous production of endothelial angiogenic agents, VEGF and bFGF by HMEC-1. In contrast, it is possible that either a low concentration or no VEGF and bFGF were produced by TIME spheroids. A previous report

showed that HMEC-1 but not HUVEC is capable of secreting VEGF and a two-fold higher level of bFGF was detected in HMEC-1 as compared with HUVEC.[10] Hence, this supports our idea that endogenous production of VEGF and bFGF by HMEC-1 are sufficient to induce invasion of HMEC-1 in 3D cultures. In this study, HMEC-1 spheroid was cultured in medium containing 10% FBS, whereas TIME spheroid was cultured in medium containing 5% FBS. It is unclear whether the concentration of FBS enhances spheroid invasion and further investigation is required.

According to a previous study, the difference in angiogenic activity of HUVEC and HMEC-1 are not associated with the different media used.[10] We cultured HMEC-1 and TIME in the same type of medium. However, we observed that HMEC-1 did not survive when cultured using EGM™-2-MV media and similarly, TIME failed to survive in MCDB131. Hence, we finally decided to use optimal media which is EGM™-2-MV for TIME and MCDB131 for HMEC-1 as recommended. In this study, a 3D-spheroid invasion assay was established with two endothelial cells as it is possible that the effects of antiangiogenic or angiogenic agents to be tested in the future could be due to different cell origin[10] or immortalizing methods. The advantage of using TIME and HMEC-1 is that these two immortalized microvascular endothelial cells retain the characteristics of the original cell line, and hence, will greatly facilitate the study on human endothelial cell biology *in vitro*.[16,18]

In this study, TIME but not HMEC-1 spheroids require the addition of VEGF and bFGF for enhancement of invasion. It has been reported that HMEC-1 is able to produce high levels of endogenous growth factors, such as VEGF and bFGF. Hence, HMEC-1 spheroid assay is not a suitable model for testing the potency of new angiogenic agents (such as for treatment of atherosclerosis) because the assay may be influenced by endogenous growth factors. The advantage of using TIME instead of HMEC-1 is that TIME can be directly used to test the potency of angiogenic growth factors whereas with HMEC-1, the activities of the endogenous growth factors produced need to be inhibited. In addition, once invasion of TIME spheroids in the 3D culture system are enhanced by VEGF and bFGF, the effect of drugs such as small molecule inhibitors targeting this specific growth factor can be tested for potential anti-cancer agents. The efficacy of drug delivery on growth factor-induced invasion in this 3D culture system can then be conducted. This is in contrast to HMEC-1 as efficacy of drug delivery may be affected by different concentrations of endogenous growth factors produced by each spheroid. It has been reported that tubule formation occurs with co-culturing of TIME cells with human glioblastoma cell line, U251.[16] Therefore, our 3D spheroid invasion assay with TIME will potentially be useful for testing tumour-induced invasion with the advantages of a 3D culture system.

The advantage of the 3D culture system compared to 2D culture system is that cells grown as 3D multicellular spheroids recapitulate the *in vivo* structure of tissues.[19] Moreover, this multicellular spheroids are able to re-establish specific biochemical and morphological features similar to the corresponding tissue.[20] This is in contrast to the 2D culture system where interaction between cells is poor.[21]

Conclusion

This is the first report on the establishment of a 3D-spheroid invasion assay with TIME cells. In addition, we have discovered that for invasion by TIME 3D-spheroids, exogenous angiogenic growth factors, VEGF and bFGF are required. It is likely that endogenous production of VEGF and bFGF by HMEC-1 are sufficient to induce invasion of HMEC-1 spheroids in 3D culture but no significant invasion was observed in TIME spheroids without exogenous angiogenic factors, VEGF and bFGF. The requirement of VEGF and bFGF for TIME spheroids invasion is a novel finding. In addition, this assay offers an advantage over HMEC-1 for testing novel angiogenic agents since it is not affected by endogenously secreted growth factors. Thus, TIME could potentially be useful for testing the potency of newly found angiogenic growth factors. Moreover, this finding will be useful for further 3D invasion studies aimed at elucidating the mechanisms underlying tumour-induced invasion of microvascular endothelial cell by VEGF and bFGF.

Competing interests
The authors declared no competing interests.

Authors' contributions

Heng Fong Seow supervised, designed the study and also edited the manuscript. Wai Kien Yip assisted in the design of study, co-ordination of technical application and statistical analysis. Norhafizah Mohtarrudin assisted as a co-supervisor in this study. Chin Tat Ng conducted the experiment, performed statistical analysis and prepared the draft.

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