Comparing Various HUVEC Cell Densities on Vascularization in a Fibrin Gel-Based Endothelial Tissue Model

Zan Chaudhry Partner: Eric Simon

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Introduction

In vitro human tissue models are instrumental for the advancement of medicine. Tissue models provide a representative ex vivo environment to study tissue properties, test therapeutics, and engineer tissue constructs for repair and replacement *in vivo*.[1] A particular field of interest is modeling the endothelium, due to the ubiquitous presence of vasculature throughout the body and the specialization of diverse endothelial tissues, such as filtering in the kidney and nutrient uptake in the gut.[2,3] The development of *in vitro* endothelial tissue models involves isolating suitable endothelial cells and creating culture conditions that mimic *in vivo* conditions.[1,2,3]

The first isolated endothelial cells were HUVECs (human umbilical vein endothelial cells), which demonstrate vast potential for vasculogenesis (the formation of vessels *de novo*) due to their fetal origin.[4,5] Vasculogenesis involves the formation of a 3D structure, requiring a suitable 3D matrix to organize cell growth. Fibrin is a natural scaffold material found predominantly in blood clotting, where native fibrinogen is polymerized into a hydrogel by thrombin to seal wounds. The same mechanism can be utilized *ex vivo* to produce 3D-organized HUVEC cultures.[3,5] Thus, HUVEC-fibrin cultures can form complex vascular networks that are representative of *in vivo* vasculature. However, HUVECs possess several limitations, particularly their origin as primary (donor-derived) cells, which increases costs and reduces availability since they cannot be expanded as widely as immortalized cell lines.[5] Thus, optimization of HUVEC cultures is a necessary step to maximize the utility of these limited cells.

The following report aims to aid in this optimization by investigating the impact of varying seeded cell count on vascularization in a HUVEC-fibrin endothelial tissue model. Three cell densities in fibrin gel are assessed: 5×10^4, 5×10^5, and 5×10^6 cells/mL. The researchers hypothesize that the 5×10^6 cells/mL group will display the greatest extent of vascularization and that increasing cell count in general will increase the extent of vascularization, as measured by an increase in the average number of branches per contiguous vessel system, the average branch length, and the percentage of lumen-containing (functional) vessels, all due to the increased availability of cells with which to form complex vascular structures.

Methods

Cell Line Details

HUVEC (Lonza; Basel, Switzerland) cells were grown in EBM2/BulletKit HUVEC media (Lonza; Basel, Switzerland), with penicillin and streptomycin, incubated at 37 ℃ / 5% CO_2.

Cell Seeding

HUVECs were cultured in a 96-well plate. Seeding information is shown in Table 1. Cells were centrifuged from HUVEC stock solution (2×10^6 cells/mL), resuspended in fibrinogen (10 mg/mL in PBS), and added to wells containing thrombin (1 U/mL in PBS). Care was taken to prevent bubble formation. However, a bubble was formed in one of the 5×10^6 cells/mL wells. Fibrin gels were set at room temp for 10 min. 200 μL of media was then added to each well, followed by incubation for 72 hours until first analysis and 96 hours until second analysis.

Condition (cells/mL):	Total Cell Count:	Total Stock Cells Volume:	Total Fibrin Gel Volume:	Fibrinogen + Cells / Well:	Thrombin / Well:
5×10^4	9×10^3	4.5 μL	150 μL	45 μL	5 μL
5×10^5	9×10^4	45 μL			
5×10^6	9×10^5	450 μL			

Table 1: Cell seeding information. Each condition was plated in triplicate, with quantities labeled "Total" indicating volumes used for the entire condition and quantities labeled "/ Well" indicating volumes used for single wells. Target cell counts were calculated by multiplying desired concentration in fibrin by desired gel volume, then by 1.1 (for fibrinogen to fibrin dilution factor), and finally by 1.1 again (to account for fibrinogen pipetting error). Gels were all 50 μL, 90% fibrinogen, 10% thrombin.

Analysis

Analysis was performed twice to assess the change in vascularization over time. Media was aspirated and cells were stained with 100 μL per well of a live/dead working solution consisting of 4 μM Calcein AM (diluted from a 4mM stock) and 4 μM EthD-1 (diluted from a 2 mM stock) in HBSS. The plate was covered with foil to prevent light exposure. Stained cells were incubated at 37 ℃ for 45 minutes. Stain was removed and wells were washed with HBSS. Fresh HBSS was added for imaging. A representative slice of each condition was captured with GFP filter (excitation/emission: 470/525 nm) to illuminate live cells/calculate vasculogenesis metrics. Images were processed by adjusting brightness/contrast, subtracting background, manually removing out of focus elements, converting to binary, manually filling in lumen, median filtering (denoising), and skeletonizing. Branch number and length were quantified with the Analyze Skeleton function in Fiji.[6] Single cell diameters were measured/averaged and a branch length threshold of 22.5 pixels (14.0 μM, similar to the literature[7]) was adopted to exclude them from analysis. The percentage of lumen-containing vessels was calculated by manually inspecting the original image for 50 randomly chosen vessel branches that had been annotated on the skeleton. Vessels with dark centers were considered lumenized. The same slices were imaged with Texas Red (excitation/emission: 585/628 nm) to illuminate dead cells.

Reagents and Equipment

Except where otherwise stated, reagents/materials were purchased from Thermo Fisher Scientific (Waltham, MA). An EVOS M5000 (Life Technologies; Waltham, MA) was used for microscopy. Fiji (ImageJ ver. 1.54b) was used for image analysis.[6]

Results

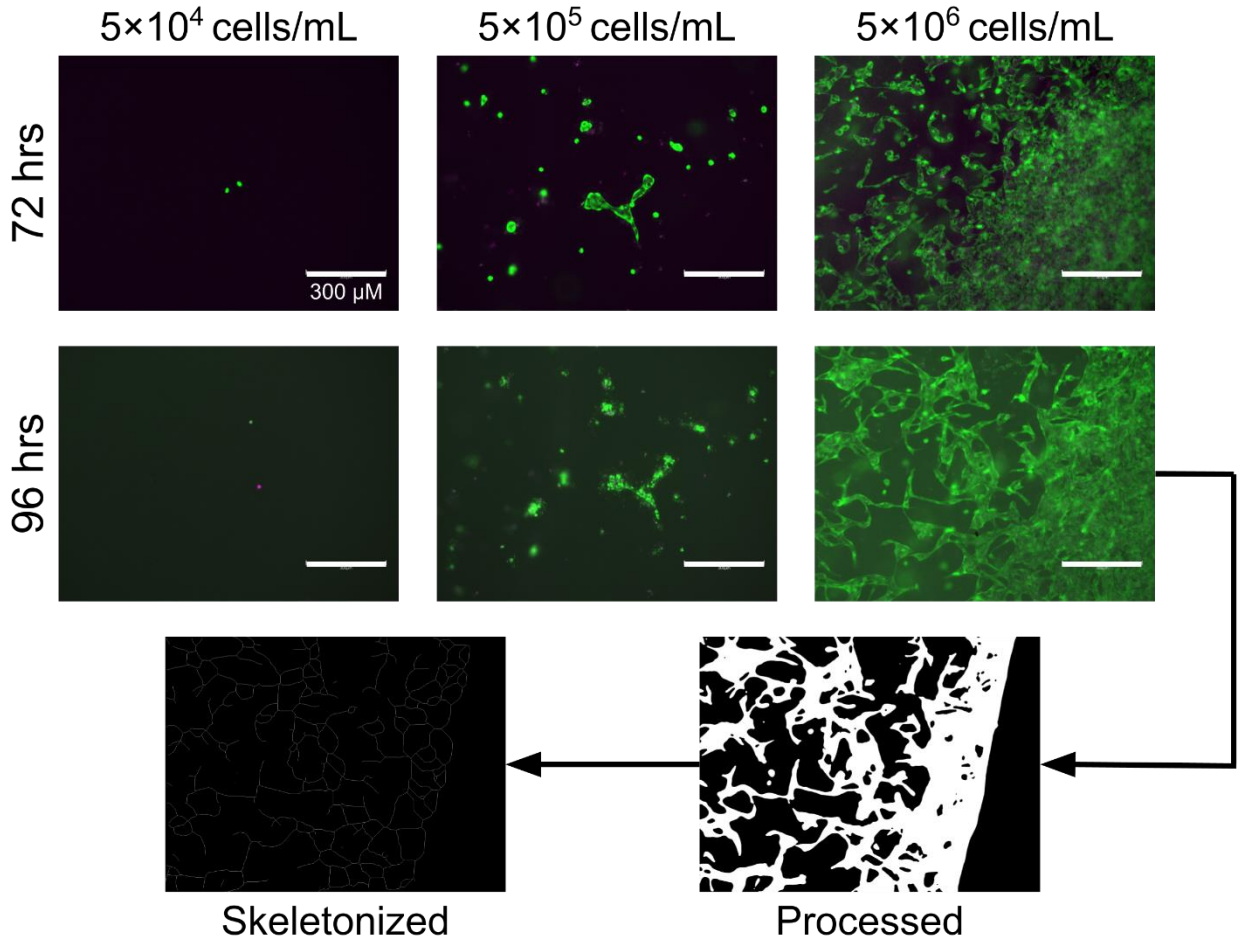

Figure 1: Composite microscope images for each condition and sample image processing. Green channel corresponds to GFP filter (highlighting live HUVECs) and red/pink channel corresponds to Texas Red filter (highlighting dead HUVECs). Scale bar is 300 μM in all images. Arrows illustrate sample image processing for quantitative vascularization metrics. Note that regions of sheet-like endothelial growth were removed from analysis, as these do not represent the desired vessel-like outcome.

Composite microscopy images (Fig. 1) illustrate HUVEC growth for all groups. The 5×10^4 cells/mL group (Group A) displayed sparse, individual cells with no vascularization. The 5×10^5 cells/mL group (Group B) displayed a mixture of individual cells, small cell clumps, and the start of a simple vascular network. The 5×10^6 cells/mL group (Group C) displayed two distinct regions of HUVEC growth: a complex vascular network and a dense sheet of cells. Lumen is visible in both Groups B and C at both timepoints. Between 72 and 96 hours, Group A displayed a marked increase in dead cells, Group B displayed minimal change, and Group C displayed an increase in connectivity (a much larger contiguous network and fewer individual vessels). Beside Group A (which contained very few cells to begin with), minimal cell death was observed, and cells displayed a healthy, elongated morphology, though with potential overcrowding in Group C. Additionally, the Group C well pictured in Fig. 1 contained a bubble within the fibrin gel that disrupted HUVEC growth in a corner region of the well (not pictured).

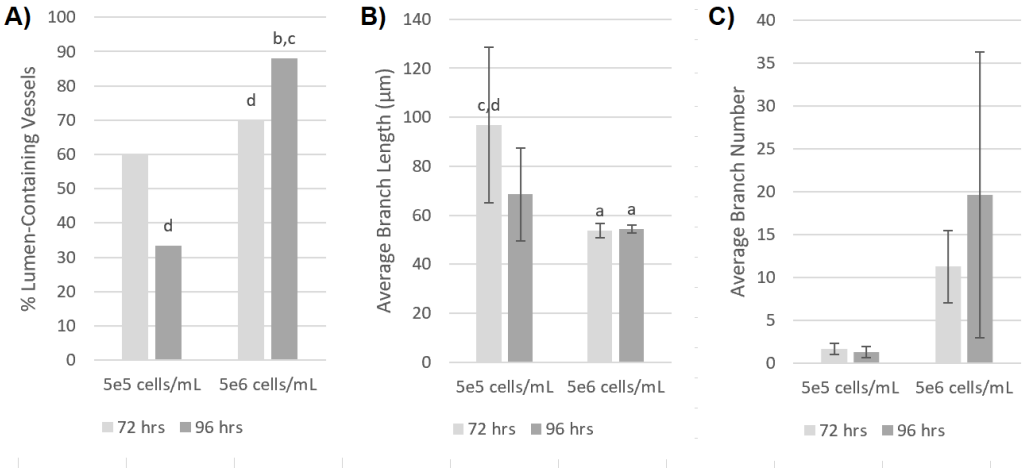

Figure 2: Quantitative metrics of vascularization. Columns are labeled alphabetically from left to right for significance testing. Letters above columns indicate a statistically significant difference between the given column and those indicated by the letters. The 5e4 group (Group A) is excluded since no vascularization was observed. Bars indicate standard error of the mean (SEM). **A)** Percentage of lumen-containing vessels for conditions that contained vascularization. Since this is a binary variable, Fisher's exact test was used for significance testing with $p < 0.05$. **B)** Average branch length in μm. Notice the high variability for the 5e5 groups. Significance tested with t-test, $p < 0.05$. **C)** Average branch number for a contiguous vascular segment. Notice the very high variability of the 5e6 groups. No significance found by t-test.

Vascularization metrics (Fig. 2) illustrated a trend (with some significance) of increasing lumen-containing vessel percentage (Fig. 2A) with increasing cell density. With increasing time, Group B lumenization decreased (insignificant) and Group C lumenization increased (significant). Average branch length (Fig. 2B) displayed a decreasing trend (with some significance) with increasing cell density, with insignificant differences between timepoints for both groups. Notably, Group B displayed high variability at both timepoints, largely due to the small number of vessels present (see Fig. 1). Finally, the average number of branches (Fig. 2C) displayed an increasing trend (though insignificant) with increasing cell density. Notably, Group C displayed high variability at both time points due to the presence of a few large networks (as high as 319 branches) and many small networks (< 20 branches). Group A was excluded from displayed data due to the lack of vascularization but can be assumed to have no expression of any of these metrics (0% lumenization, 0 μM branch length, 0 branches), which contributes to the observed trends with respect to cell density for lumenization and branch number but detracts from the trend in branch length.

Discussion

Prior to the experiments, it was hypothesized that increasing cell density would increase the extent of vascularization in a HUVEC-fibrin tissue model, as measured by lumenization (functionality), average branch length (growth), and average branch count (complexity). The data largely supports this hypothesis. Visual inspection of microscopy images (Fig. 1) illustrates a clear trend towards increasing prevalence of vessel-like structures and increasing connectivity of these vessels (longer contiguous networks) with increasing cell density. Quantitative metrics (Fig. 2) mostly support these observations, with increases in

lumenization and branch number with increasing cell count. Average branch length, however, displayed an interesting trend, increasing from the 5×10^4 cells/mL group (Group A, which displayed no vascularization) to the 5×10^5 cells/mL group (Group B), but decreasing from Group B to the 5×10^6 cells/mL group (Group C). Likely, this pertains to the types of networks present in Groups B and C. In Fig. 1, Group B clearly contains a single, large (lengthwise, with branches spanning ~150 μm) and simple (only 3 branches) network while Group C contains a vast (up to 319 branches) network of vessels of all sizes (from ~30 -300 μm). Only one of the Group B wells contained vasculature and this well was used for calculations. The lack of vasculature in Group B limits observations to the single network present, which likely artificially inflates the average branch length. A future study could investigate a larger number of samples prepared with this cell density for a more accurate account of the extent of vascularization.

Interesting trends were observed between timepoints, most notably a trend towards decreasing lumenization, branch length, and branch count in Group B with increasing time. The images in Fig. 1 provide some insight. At 72 hours, the single developing network and some small cell clumps/buds are visible, while at 96 hours, some of the clumps/buds have matured into larger structures, though not nearly to the extent of the network. These larger clumps/buds were included in analysis, and thus deflated the (as previously discussed) artificially inflated metrics for Group B at 72 hours. A future study could investigate over a longer time course to monitor the maturation of these growing clumps/buds. Group C displayed the expected (per the literature[8]) trend of increasing lumenization, branch length, and branch count over time.

Another point of interest was the formation of a sheet of HUVECs in one of the Group C wells (Fig. 1). This growth was not observed in any other condition or well but has been observed in the literature.[7] HUVECs are adherent cells and will form a sheet without a 3D scaffold such as fibrin, so this outcome suggests some disruption in the fibrin gel, which was observed. The well contained a bubble that seemingly interfered with the growth pattern, with sheet-like growth concentrated near the bubble. However, this well also exhibited the most complex vascular network formation, with an average branch number of 46.7 compared to 3.4 for the other Group C well that was analyzed, raising questions about how mechanical disturbances/fibrin structural changes impact vascularization, which could form the basis of a future study.

The presented experiments measured the effect of varying HUVEC density on vascularization in a HUVEC-fibrin endothelial tissue model. The results suggest increasing vascular complexity/functionality with increasing cell density (agreeing with the literature[8]). Overall, this report provides guiding information for optimizing HUVEC-fibrin tissue models and advance modeling capabilities to increase understanding of the tissue, better test therapeutics, and improve regenerative medicine potential.

References

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