Published in final edited form as:

Cell Mol Bioeng. 2010 March ; 3(1): 60–67. doi:10.1007/s12195-010-0112-4.

Assembly of Human Umbilical Vein Endothelial Cells on Compliant Hydrogels

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Abstract

Angiogenesis is the process by which endothelial cells grow and disassemble into functional blood vessels. In this study, we examine the fundamental processes that control the assembly of endothelial cells into networks in vitro. Network assembly is known to be influenced by matrix mechanics and chemical signals. However, the roles of substrate stiffness and chemical signals in network formation is unclear. In this study, human umbilical vein endothelial cells (HUVECs) were seeded onto RGD or GFOGER functionalized polyacrylamide gels of varying stiffness. Cells were either treated with bFGF, VEGF, or left untreated and observed over time. We found that cells form stable networks on soft gels (Young's modulus 140 Pa) when untreated but that growth factors induce increased cell migration which leads to network instability. On stiffer substrates (Young's modulus 2500 Pa) cells do not assemble into networks either with or without growth factors in any combination. Our results indicate that cells assemble to networks below a critical compliance, that a critical cell density is needed for network formation, and that growth factors can inhibit network formation through an increase in motility.

Keywords

Polyacrylamide gel; Substrate Stiffness/Compliance; bFGF; VEGF; Angiogenesis

INTRODUCTION

Angiogenesis is the sprouting of microvessels to form new capillary networks. Angiogenesis occurs by endothelial cell migration away from existing vasculature after degradation of the surrounding basement membrane, and endothelial cell proliferation. The endothelial cells form a new capillary lumen, and after the synthesis of new basement membrane, supporting cells like pericytes and smooth muscle cells are recruited to stabilize the new vessels.^{13,24} Determining the mechanism of angiogenesis is important because it is linked to wound healing, morphogenesis, tumor formation and metastasis, and is important for tissue engineering. Endothelial cells not only help form new blood vessels; they also serve an important function as a physical barrier between the blood and other tissues and they help maintain vascular homeostasis.¹⁴

Substrate stiffness is a proven regulator of cell behavior and adhesion.¹⁰ It has been shown that compliant substrates promote assembly of bovine aortic endothelial cells into networks in 2D⁶ and endothelial cell tubulogenesis in 3D,^{8,9} and that substrate stiffness affects the type and method of tube formation in 3D.^{28, 31} A variety of micropatterning techniques,

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growth factors, and adhesion molecules have been used to induce endothelial cells to form these capillary-like tubes^{9,26,27} in vitro. Two of the growth factors often associated with angiogenesis are bFGF and VEGF. Tyrosine kinase receptors for VEGF^{4,5} and FGF³ are found on endothelial cells, and both VEGF and FGF have been implicated in inducing angiogenesis in vivo.^{1,4,13,24} Tissue formation may also be controlled by the balance between cell-cell adhesions and cell-substrate adhesions.^{12,21,25} The effect and interplay of growth factors, matrix mechanics, and adhesions on network formation and cell migration is still uncertain.

We have investigated the effects of matrix mechanics and chemical signals on endothelial cell migration and network assembly in this study. Specifically, human umbilical vein endothelial cells (HUVECs) were seeded on RGD peptide-functionalized polyacrylamide gels of varying stiffness. RGD is a fibronectin peptide that binds $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins.^{11, 16} Polyacrylamide gels facilitate simple manipulation of substrate stiffness by altering the amount of bis-acrylamide used to cross-link each gel.³⁰ Cells were treated with both bFGF and VEGF to determine how chemical signals often associated with angiogenesis affect endothelial cells. Additionally, to determine if matrix mechanics affect different integrins HUVECs were seeded onto polyacrylamide gels functionalized with GFOGER, a collagen peptide that integrin $\alpha_2\beta_1$ binds.²³ Our results indicate that HUVECs assemble into networks on compliant surfaces but not stiff surfaces and that growth factors disrupt network formation and increase cell migration on soft surfaces.

MATERIALS AND METHODS

Coverslip Activation

Coverslips were activated using previously described methods.⁷ Briefly, circular glass coverslips (No. 1, 22 × 22 mm, Fisher Scientific, Pittsburgh, PA) were incubated in 0.2 M HCl overnight. The coverslips were then rinsed with deionized water and incubated with 0.1 M NaOH. After washing with deionized water, the coverslips were incubated in 0.5% (v/v) 3-aminopropyl-trimethoxysilane (Sigma-Aldrich, St. Louis, MO) in water for 30 minutes. After washing thoroughly in deionized water, the coverslips were incubated in 0.5% (v/v) glutaraldehyde (70% aqueous stock solution, Sigma-Aldrich, St. Louis, MO) in PBS for one hour. The coverslips were then rinsed in deionized water a final time and allowed to dry.

Polyacrylamide Gel Synthesis

Gels were prepared using varying ratios of acrylamide to bis-acrylamide in the solution to make gels of varying Young's moduli as previously described.^{17,22} Briefly, gels were prepared with 5% - 3% acrylamide (40% w/v solution), 0.2% - 0.4% bis-acrylamide, 0.05% n'-tetramethylethylenediamine (Bio-Rad, Hercules, CA) and 0.25 M HEPES. The gel solutions were adjusted to pH 6.0, and then 5.6 mg N-6-((acryloyl)amido)hexanoic acid (N-6) dissolved in ethanol was added per ml of gel solution. N-6 was synthesized using the method specified by Pless and co-workers¹⁹ in our lab. The solution was degassed for 30 minutes, and then polymerization was initiated with 6 μ l of 10% ammonium persulfate per ml of gel solution. After mixing, 20 μ l was deposited onto the activated coverslip and then sandwiched under a RainX® (Sopu Products, Houston, TX) coated 18 × 18 mm circular coverslip (No. 1, Fisher Scientific, Pittsburgh, PA). Gels were allowed to polymerize for 45 minutes in a bag purged of oxygen with nitrogen. Circular coverslip were removed, and after rinsing with MilliQ water the gels were incubated with 0.1 mM RGD or 0.1 mM GFOGER overnight at 4°C.

The amount of peptide bound to the gel can be controlled by the concentration of peptide in the solution applied to the gel surface.²¹ Any unbound N-6 linker remaining after the

peptide incubation was reacted with 0.1% ethanolamine (Sigma-Aldrich, St. Louis, MO) in 50 mM HEPES (pH 8.0, Sigma-Aldrich, St. Louis, MO) for 30 minutes. Gels were then washed with MilliQ water and stored in PBS or assembled into 6 or 12 well plates. The gels were held in place in the plates with vacuum grease and then UV sterilized in EGM media (Lonza, Walkersville, MD) for 30 minutes.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in EGM Endothelial Growth Media (Lonza, Walkersville, MD) supplemented with 0.4% bovine brain extract (BBE) with heparin, 0.1% h-EGF, 0.1% hydrocortisone, 0.1% gentamicin sulfate (GA-1000), and 2% fetal bovine serum (FBS). Cells were maintained in plastic culture flasks at 37°C in a humidified atmosphere containing 5% CO₂ in air and subcultured when the flasks were 70% to 90% confluent. HUVECs were used between passages 5–7.

HUVEC Network Assembly

HUVECs were plated on gels with various Young's moduli (140 – 2500 Pa) at densities of 100,000 – 200,000 cells per well of a six-well plate. Immediately after plating cells, 5 locations per gel were chosen and time-lapse imaging began. For those experiments with growth factors (bFGF and VEGF), the cells were preincubated in 1 nM growth factor for 20 minutes before plating on gels, and they were plated with and maintained in 1 nM of the desired growth factor for the remainder of the experiment. A growth factor concentration of 1 nM was chosen as it had previously been shown to be in the range for maximum invasion of endothelial cells for *in vitro* angiogenesis assays.¹⁸ A Nikon Eclipse TE300 microscope was used to capture cell images with 10× magnification. All cell imaging was performed at 37°C and 5.0% CO₂. For cell-assembly studies, time-lapse images were taken every 10–30 minutes. ImageJ plugins Manual Tracking and Chemotaxis Tool were used to determine HUVEC velocity. ImageJ and both plugins are freely available through the NIH website.²⁰ Cells were imaged for up to 34 hours after seeding for the growth factor studies and analyzed through two separate time periods. The cells were tracked during these two different time periods to determine both if cells decreased their velocity after network formation was begun and if exposure to growth factors over time altered cell velocity.

RESULTS

Compliant substrates promote HUVEC network assembly

In order to investigate the interplay between substrate stiffness and endothelial cell network assembly, we cultured HUVECs on polyacrylamide gels with Young's moduli of 140, 675, 1050, and 2500 Pa. All of the gels were functionalized with 0.1 mM RGD to ensure that ligand density did not influence cell behavior. Polyacrylamide gels by themselves are inert to cell adhesion and ligand adsorption, so the peptide concentration remains constant over time and amongst different gels.¹⁷ Therefore, any changes seen in cell behavior can be attributed to substrate mechanics only. On the softest gels, 140 and 675 Pa, HUVECs organize into 2D networks as seen in Figure 1a and 1b. These networks are typified by cells connecting into 2D circular structures and forming cords that connect groups of cells. In Figure 1c at 1050 Pa, the cells appear to be in an intermediate state with some cells connecting to one another in network-like structures and other cells spreading and growing in patches similar to what is seen in Figure 1d on a 2500 Pa substrate. At 2500 Pa, the cells are more spread than cells on softer substrates, and these cells grow in patches to confluence rather than forming stable networks. Cell area increases with increasing stiffness for each of the Young's moduli tested (figure 1e). Substrate stiffness, independent of differences in growth factors or adhesion ligand, affects both HUVEC organization and cell spreading.

Substrate stiffness was also found to affect HUVEC organization when acting through collagen receptors when the polyacrylamide gels were functionalized with 0.1 mM GFOGER peptide. Figure 1f shows how HUVECs again organize into a 2D network when plated on 140 Pa gels and in figure 1g they are much more spread, disorganized, and grow in clusters on the 2500 Pa gel. The network assembly on soft gels and the lack of organized assembly on stiffer gels is similar when HUVECs attach to a RGD coated substrate through $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins or a GFOGER coated substrate through $\alpha_2\beta_1$ integrins.

Effect of cell number on network formation

As seen in Figure 1, the most defined endothelial cell network formed on the softest gel, 140 Pa. Even on the 140 Pa gel, however, we found that a minimum number of cells is required for network assembly. As shown in Figure 2a, when a low density of cells is plated, 7,000 cells per cm^2 , the HUVECs barely move or crawl and do not form a network. At 10 hours the cells, rather than forming cords, simply stay separate or condense into islands of cells to maximize the cell-cell contacts available to them and do not form a network. Figure 2b shows that at 11,000 cells per cm^2 , the cells are at a sufficiently high density so that when they spread, they can stretch and reach for their neighbors and the beginnings of a sparse network. Initial network connections are made, and cords begin to form. At higher densities such as 13,000 cells per cm^2 , shown in Figure 2c, cells move more readily and form a more defined network. Specifically, the cells were seeded in a random manner but still separate into cords and 2D circular structures to form a network on the 140 Pa gels. Viewed together, Figure 2 shows that increased cell density leads to increased cell movement (Figure S1) and increased network formation.

bFGF and VEGF on Network Formation

bFGF and VEGF have both been implicated in inducing angiogenesis,¹⁸ and it has been shown that endothelial cells migrate chemotactically in gradients of FGF2 and VEGF.² It is unknown, however, if the effectiveness of these growth factors is influenced by substrate stiffness or if matrix mechanics and chemical signals are independent controllers of endothelial cell migration and network assembly. Figure 3 shows how stimulation of HUVECs with bFGF or VEGF affects network formation on soft substrates, and Figure 4 shows how the same stimulation affects network formation on stiff substrates. Cells were preincubated with 1 nM growth factor for 20 minutes before plating 15,000 cells per cm^2 on gels (140 Pa or 2500 Pa), and they remain in 1 nM of the desired growth factor for the entire sequence. In Figure 3a, one can see that without growth factors, a stable, constant network forms and becomes increasingly refined with time. The addition of either of these growth factors, however, changes the network formation. When bFGF is added to the system (Figure 3b) the HUVECs still spread and initially begin to form networks in a similar way to those seen in the control experiments performed without growth factors; networks with circular groups connected by cords are again observed. The networks formed in the presence of 1 nM bFGF are unstable; specifically, the networks continually shift and modify into new network assemblies with time. The same effect is seen in Figures 3c and 3d; 1nM VEGF treatment and combination treatment of 1 nM bFGF and 1 nM VEGF, respectively, result in unstable networks. This instability is shown in Figure 3 and more clearly in Supplemental Movie 1 where untreated HUVECs, HUVECs treated with bFGF, VEGF, or bFGF and VEGF on 140 Pa substrates generally move with other cells. They either crawl by attaching to and following a neighbor, by crawling along other cells, or by stretching and moving from one neighboring cell to another.

Figure 4a shows that on stiffer substrates (2500 Pa), HUVECs do not form ordered network structures even up to 30 hours after cell seeding. Figures 4b, 4c, and 4d show HUVECs on 2500 Pa substrates stimulated with 1 nM bFGF, 1 nM VEGF, and the combination of 1 nM

bFGF and 1 nM VEGF, respectively. On this stiffer substrate where network formation is not seen in the absence of growth factor, the addition of growth factors does not affect the assembly of endothelial cells. Growth factors do not induce network formation on 2500 Pa gels, and the cells migrate independently of their neighbors both with and without growth factors as shown in Figure 4. This result can also be seen in Supplemental Movie 2.

Additionally, the effect of these growth factors on cell migration velocity on both soft and stiff gels was determined by manually tracking the cells movements in Supplemental Movie 1 and 2. Figure 5 shows that on stiffer substrates (2500 Pa), cells crawl and migrate more quickly than cells in similar conditions on softer substrates (140 Pa). The addition of growth factors has a larger impact on the migration speed of cells on softer 140 Pa gels than they do on cells plated on stiff substrates (Figure 5). Control cells without additional growth factor on 140 Pa substrates slowed down significantly after 18 hours, once a stable network was formed. The three different growth factor conditions tested, 1 nM VEGF, 1 nM bFGF, and the combination of 1 nM VEGF and 1 nM bFGF, significantly increased the cell velocity over untreated cells on 140 Pa gels. Thus, the growth factors affected cells early, and the cell speed did not decrease after the first 18 hours as it did with untreated cells. This increase in cell velocity led to the disassembly of the stable cell networks. Interestingly, the combination of VEGF and bFGF did not increase cell velocity compared to cells treated with only one growth factor. Other studies, however, have shown that the combination of VEGF and bFGF has an additive or synergistic effect on angiogenesis and tubule sprout length both *in vitro*¹⁸ and *in vivo*.¹ The studies that demonstrate a synergistic effect of bFGF and VEGF were done on 3D collagen matrices¹⁸ and in a 3D *in vivo* environment,¹ so this synergistic effect may only occur in 3D cultures or on gels of different materials. In our experiments, the cells stimulated with VEGF or VEGF plus bFGF did not significantly change velocity from the first 18 hours to 18 – 34 hours after seeding, but the cells with just bFGF treatment on 140 Pa gels did significantly increase their velocity over time.

On 2500 Pa substrates, neither the addition of VEGF nor the addition of bFGF caused a significant increase in cell velocity compared to untreated cells (Figure 5). The migration speeds of untreated cells on 2500 Pa gels did not decrease after 18 hours as they did on the 140 Pa gels. Similarly, just as the growth factors did not change the early migration velocity compared to untreated cells on 2500 Pa, those cells treated with bFGF or VEGF plus bFGF did not change their velocity from the first 18 hours to 18 – 34 hours after seeding. The HUVECs treated with bFGF on 2500 Pa gels did, however, significantly increase their velocity after the first 18 hours.

DISCUSSION

HUVECs form networks on soft substrates and grow into confluent monolayers on stiff substrates. Figure 1 indicates that at a compliance of approximately 1000 Pa the behavior switches from where networks form to where they do not form. Cells have been shown to spread more on stiffer substrates (figure 1e) even with the same ligand concentration,¹⁰ and it has also been shown that cells on stiffer substrates move more quickly and that cell pairs move more slowly than single cells.²¹ There is a correspondence between cell spreading and substrate adhesiveness, and the spreading of a population of cells was shown to be a competition between cell-cell adhesion and cell-substrate adhesion.²⁵ Increased cell-cell adhesions through cadherins has also been shown to decrease the invasiveness of malignant cells into matrigel.²⁹ These results together indicate that a balance exists between cell-substrate adhesions and cell-cell adhesions in affecting the extent of cell spreading and cell migration and that these adhesions are subject to matrix mechanics. Similar results were found in this study, but this study was extended to also analyze cell network formation. Reinhart-King and co-workers found that bovine aortic endothelial cells assemble into

networks on soft gels with sufficient ligand, on stiff gels with less available ligand, and that fibronectin polymerization stabilizes cell-cell contacts and is required for assembly.⁶ We extended this work to show that HUVEC assembly is also dependent on substrate compliance when sufficient ligand is available, a minimum cell density is required for assembly, and showed that the addition of growth factors can increase cell migration and disrupt assembly. On softer gels in which cells are less spread, fewer cell-substrate adhesions are formed thus cell-cell adhesions are dominant; this result explains why stable cell networks form on soft gels. This endothelial cell network formation led to slower cell velocities because movement is inhibited by cell-cell adhesions. Cell-cell contacts are less important on stiffer gels because more cell-substrate adhesions form.¹⁷ Fewer, weaker cell-cell contacts lead to faster migration that is independent of neighboring cells (Supplemental Movie 2). Additionally, a minimum number of cells are required for network formation because the decreased migration on soft gels hinders the ability to find neighbors to form connections. Endothelial cells can communicate through substrate strains caused by the traction stresses of neighboring cells.²¹ If cells are too sparse, then they may not be able to sense the traction stresses of their neighbors or they may not sense any autocrine signaling that may occur, and in turn, they will not crawl the long distances required to contact other cells.

We are currently developing quantitative tools for characterizing the assembly of networks. One way would be to calculate a radial distribution function, $g(r)$, for the probability of finding a cell at a certain distance from any other cell. Such a tool is often used to examine the structure of multi-particle systems.¹⁵ We have found that given the size of the field of view, we cannot yet get sufficiently good statistics to use $g(r)$ in a meaningful way. However, by examining the ratio of cell length to cell width, which is a simple metric of cell elongation, we could determine a correlation that reflects the assembly of the population. As shown in figure 6, examining data for HUVEC assembly on RGD surfaces at an initial cell density of 15,000 cells/cm², we found that this ratio is correlated with the propensity of cells to form networks.

We also showed that on soft gels the growth factors bFGF and VEGF increased cell migration and resulted in transient network formation and frequent structural rearrangement. The cells continually moved in and out of new arrangements and were never able to settle into a stable arrangement. The combined treatment of bFGF plus VEGF on 140 Pa gels did slightly decrease cell velocity with time (Figure 5) and this network appears to be the most stable of those treated with growth factor (Supplemental Movie 1). These results indicate that HUVECs move more quickly when they are trying to move into or find a network. Once they are in an assembled formation, the cells may move within the network and continue probing for possible new connections, but they appear to be in a more stable environment because they do not crawl as far or as quickly as before (Figure 5).

Previous studies show that endothelial cells will migrate towards FGF and VEGF gradients at similar levels to those used in this study.² Chemotaxis of those endothelial cells, however, is severely diminished by high levels of FGF and VEGF in a linear gradient. This finding led Barkefors and co-workers to propose that endothelial cells will initially migrate in a gradient of FGF or VEGF. As the cells get closer to the source of the growth factor, at the high end of a chemotactic gradient, they will shift to a non-migrating phenotype.² Thus a chemical gradient could be the signal for cells to sprout from existing vessels and begin to form new microvessels. Once the microvessel extends to the source of the gradient, the location needing more blood flow, the cells would stop migrating, and the vessel would stabilize. Our data agrees with this hypothesis; in the presence of an intermediate concentration of growth factor, HUVECs move out of their stable network formation and increase their migration velocity. The question remains if further increasing the bFGF and VEGF concentrations

used in these assembly studies will result in a decrease in endothelial cell migration. If cell migration decreases the cells may then form back into a network on soft substrates. This work indicates that matrix mechanics can drive network assembly by determining the number of cell-substrate adhesions, independent of ligand density, and that growth factors can drive cells to migrate out of an assembled structure. Finally, matrix stiffness can result in the same phenotype of loss of network assembly and increased cell migration as the addition of growth factors had on endothelial cells on soft substrates. These findings help explain the early stages of angiogenesis and can be used to inform the design of biomaterials for tissue engineering and angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH HL08533.

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FIGURE 1.

HUVEC network assembly occurred on soft substrates and is characterized by cells connecting into circular structures and forming cords that connect groups of cells. HUVECs were seeded on polyacrylamide gels functionalized with 0.1 mM RGD (a–e). (a) 140 Pa gel promoted network assembly (b) 675 Pa gel promoted network assembly (c) 1050 Pa is an intermediate stiffness with patches of spread cells and what appear to be some cords (d) 2500 Pa gel did not promote network assembly. (e) Cell area increases as the substrate stiffness increases. Via ANOVA, $p < 0.001$ between groups. HUVECs were seeded on polyacrylamide gels functionalized with 0.1 mM GFOGER (f–g). (f) 140 Pa gel promoted network assembly (d) 2500 Pa gel did not promote network assembly. scale bar = 100 μm

FIGURE 2.

A minimum cell density is required for network assembly. HUVECs were seeded on 140 Pa polyacrylamide gels linked with 0.1 mM RGD (a) 7,000 cells/cm² form islands of cells or stay separate and are too sparse to form a network. (b) 10,000 cells/cm² just begins to form network connections and cords. (c) 13,000 cells/cm² are dense enough to form a stable network. scale bar = 100 μ m

FIGURE 3.

FGF and VEGF disrupt network assembly on compliant substrates. HUVECs were seeded on 140 Pa polyacrylamide gels linked with 0.1 mM RGD. (a) Untreated cells form a stable network as time progresses. (b) 1 nM FGF disrupts stable network formation (c) 1 nM VEGF disrupts stable network formation (d) 1 nM FGF and 1 nM VEGF disrupt stable network formation. Time lapse images were taken every 20 minutes. scale bar = 100 μ m

FIGURE 4.

The addition of growth factor does not change HUVEC behavior on stiff substrates; a network does not assemble with or without growth factor. HUVECs were seeded on 2500 Pa polyacrylamide gels linked with 0.1 mM RGD. (a) Untreated cells do not form a network on stiff substrates (b) 1 nM FGF does not change HUVEC behavior (c) 1 nM VEGF does not change HUVEC behavior (d) 1 nM FGF and 1 nM VEGF does not change HUVEC behavior. Time lapse images were taken every 20 minutes. scale bar = 100 μ m

FIGURE 5.

HUVECs were seeded on 140 Pa or 2500 Pa polyacrylamide gels linked with 0.1 mM RGD. The cells were first tracked from 1 hour to 18 hours after seeding. Tracking began at 1 hour, so that the migration velocity was not skewed by the period when the cells were first attaching and spreading. The second tracking phase was from 18 hours to 34 hours after seeding. † indicates a significant difference between control and growth factor treated cells ($p < 0.001$), * indicates a significant difference in velocity between 1–18 hours and 18–34 hours ($p < 0.05$). Significance was determined with a Student's t-test.

FIGURE 6.

Aspect ratio, the ratio of cell length to width, is a useful metric for quantifying the propensity of cells to form networks. HUVECs were seeded onto 140, 675, 1050, or 2500 Pa polyacrylamide gels linked with 0.1 mM RGD. HUVEC network assembly occurred on soft substrates (140 Pa and 675 Pa) and is characterized by an average cell aspect ratio that is greater than 3. Network formation did not occur on stiffer gels (1050 Pa and 2500 Pa) and the average cell aspect ratio is less than 3.