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ANGIOGENIC ENDOTHELIAL CELL INVASION INTO FIBRIN IS STIMULATED BY PROLIFERATING SMOOTH MUSCLE CELLS

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Abstract

These studies aimed to determine the effect of smooth muscle cells (SMCs) on angiogenic behavior of endothelial cells (ECs) within fibrin hydrogels, an extracellular matrix (ECM) commonly used in tissue engineering. We developed a 3-D, fibrin-based co-culture assay of angiogenesis consisting of aggregates of SMCs with ECs seeded onto the aggregates' surface. Using digital fluorescence microscopy, EC matrix invasion was quantified by average length of sprouts (ALS) and density of sprout formation (DSF). We demonstrated that ECs and SMCs co-invade into the ECM in close proximity to one another. ECs that were co-cultured with SMCs demonstrated increased invasion compared to ECs that were cultured alone at all time points. At Day 19, the ALS of ECs in co-culture was 327 $\pm$ 58 μ m versus 70 $\pm$ 11 μ m of ECs cultured alone ($p=.01$). The DSF of co-cultured ECs was also significantly greater than that of ECs cultured alone ($p=.007$ on Day 19). This appeared to be a function of both increased EC invasion and well as improved persistence of EC sprout networks. At 7 days, ECs in co-culture with proliferation-inhibited SMCs previously treated with Mitomycin-C (MMC) demonstrated significantly attenuated sprouting compared to ECs co-cultured with SMCs that were untreated with MMC (82 $\pm$ 14 μ m versus 205 $\pm$ 32 μ m; $p < .05$). In assays in which multiple co-culture aggregates were cultured within a single hydrogel, we observed directional invasion of sprouts preferentially towards the other aggregates within the hydrogel. In co-culture assays without early EC/SMC contact, the ALS of ECs cultured in the presence of SMCs was significantly greater than those cultured in the absence of SMCs by Day 3 (320 $\pm$ 21 μ m versus 187 $\pm$ 16 μ m; $p < .005$). We conclude that SMCs augment EC matrix invasion into 3-D fibrin hydrogels, at least in part resulting from SMC proliferative and invasive activity. Directed invasion between co-culture aggregates and augmented angiogenesis in the absence of early contact suggests a paracrine mechanism for the observed results.

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Keywords

Smooth muscle cell; endothelial cell; pericyte; angiogenesis; co-culture; tissue engineering; fibrin; hydrogel; tissue engineered blood vessel; capillary

Introduction

Pericytes, derived from vascular mesenchymal cells such as smooth muscle cells (SMCs) and adventitial fibroblasts, surround the endothelium of capillaries and contribute to the later maturation and stabilization stages of angiogenesis. Direct contact via gap junctions, adhesion plaques, and adherens junctions, as well as indirect paracrine mechanisms between endothelial cells (ECs) and pericytes, allow for significant interactions which impact EC differentiation, migration, proliferation, and quiescence.(Gerhardt, Wolburg et al. 2000, Rucker, Wynder et al. 2000, Gerhardt and Betsholtz 2003, Tillet, Vittet et al. 2005) In addition, pericytes and their precursors appear to have a significant role in mediating the early events of active angiogenesis.(Ozerdem, Grako et al. 2001, Ozerdem, Monosov et al. 2002, Brey, McIntire et al. 2004, Kunz-Schughart, Schroeder et al. 2006) Proteins including VEGF and deposited extracellular matrix (ECM) proteins such as fibronectin secreted by SMCs and fibroblasts *in vitro* have been demonstrated to elicit angiogenic activities such as increased plasminogen activity, proliferation, motility, and cordlike structure formation in co-cultured ECs.(Montesano, Pepper et al. 1993, Kuzuya, Satake et al. 1995, Chon, Wang et al. 1997, Kale, Hanai et al. 2005, Davie, Gerasimovskaya et al. 2006, Dietrich and Lelkes 2006) Evidence that the local extracellular environment may regulate paracrine interactions between ECs and pericytes is provided by the observed endothelin-1-mediated activation of EC mitogenesis by fibroblasts,(Davie, Gerasimovskaya et al. 2006) and the production of Ang-2 and VEGF in mesangial cells (renal pericytes) under hypoxic conditions.(Yuan, Yang et al. 2000) *In vivo* observations of pericytes “leading” angiogenic sprouts and lining vascular tubes that lack ECs suggest a more direct role for pericytes as guiding cells during angiogenesis.(Nehls, Denzer et al. 1992, Ozerdem and Stallcup 2003) These data are consistent with observations that under hypoxic stimulation, VEGF-secreting perivascular cells appear to be the first vascular cells to invade the *corpus luteum* parenchyma during ovulatory angiogenesis.(Reynolds, Grazul-Bilska et al. 2000) Thus, while the contribution of pericytes to the later stabilization and maturation of neocapillaries is well established, the likelihood that they promote angiogenic induction given the proper pro-angiogenic environmental context suggests a broader role for these cells which warrants further study. (Nehls and Drenckhahn 1995)

In tissue engineering, the need for microvasculatures to supply nutrients and oxygen for constructed organs such as tissue-engineered blood vessels (TEBVs) is thought to be critical. Specifically, the utilization of mesenchymal cells such as SMCs and fibroblasts to construct the wall of a tissue-engineered blood vessel may alter the angiogenic processes within the wall of the vessel that precede TEBV vasa vasorum development. The purpose of these studies is to determine how SMCs affect the angiogenic behavior of endothelial cells within fibrin hydrogels, an extracellular matrix frequently used in tissue engineering, and a common component to hydrogel-based TEBVs. We hypothesize that SMCs will promote angiogenic activity by promoting ingrowth into a 3-D fibrin matrix. We will also test if contact between SMCs and ECs is necessary for promotion of angiogenic activity or inhibitory of SMC-mediated effects on EC angiogenic activity. We designed three unique *in vitro* co-culture models of angiogenesis in order to address these questions.

Materials and Methods

Materials

Chemicals, biological reagents, and experimental supplies were obtained as follows: collagenase (Invitrogen); human thrombin (American Red Cross; Rockville, MD); Mitomycin C, L-ascorbic acid, methylcellulose, fibrinogen, transferrin, insulin, anti- α -actin antibody and aprotinin, anti-VWF antibody, PKH-27, PKH67 (Sigma Chemical Co.; St. Louis, MO); bovine lung heparin (Upjohn; Kalamazoo, MI); 0.05% trypsin/EDTA, HBSS, M199, DMEM, L-nonessential amino acids, sodium pyruvate, penicillin, streptomycin, DMEM-F12 (Gibco, Grand Island, NY); fetal bovine serum (FBS) (Hyclone, Logan, UT); 100-mm and 60mm Petri dishes (Fisher Scientific; Pittsburgh, PA); round bottom 96 well plates (Greiner Bio-one; North Carolina); 96-well polystyrene plastic plates (Beckton Dickinson; Lincoln Park, NJ); woven nylon mesh rings (ID = 7.5 mm, OD = 13 mm) (Sefar America Inc; Kansas City, MO); Parafilm M (American National Can, Greenwich, CT); fetal bovine serum (FBS) (Hyclone, Logan, UT); 24 wells plates and tissue culture flasks, Costar Transwell polystyrene plates (Corning Costar Corp; Cambridge, MA).

Animal Care

All animal procedures complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985) and were approved by our institutional IACUC.

Cell Isolation

SMCs and ECs were harvested from adult mongrel canine carotid arteries and external jugular veins respectively per our previously published protocols. (Kang, Gosselin et al. 1995, Brewster, Brey et al. 2004) EC identity was confirmed using immunofluorescent dual staining with anti-vWF and anti- α -actin antibodies. Only EC exhibiting 95% positive vWF staining and 2% or less α -actin staining were used. ECs were used within passages 1 to 5. Primary SMCs migrating from the carotid artery explants were used in all experiments. SMC identity was confirmed by immunofluorescent staining with anti- α -actin antibodies, and only cultures exhibiting 95% positive staining were used in the assays. Individual carotid artery explants were used as the source of cells for assays no more than 3 times.

Mitomycin C Treatment

In experiments using Mitomycin C (MMC) treatment to inhibit cell proliferation, 500,000 SMCs were plated onto 60mm Petri dishes and treated with 10 μ g/mL of MMC in SMC growth media containing DMEM, 10% FBS, PCN/Strep (100 U/mL), Gentamicin (.05 μ g/mL), Amphotericin (.25 μ g/mL), non-essential amino acids, MEN, and sodium pyruvate for one hour prior to use in experiments as described in previously published methods. (Ucuzian, Brewster et al.) Preliminary experiments and previously published data demonstrated viability of cells as well as significant inhibition of SMC proliferation demonstrated by reduced thymidine incorporation and of matrix invasion when treated with this protocol. (Ucuzian, Brewster et al.) SMCs that were concurrently plated in SMC growth media, but not treated with MMC, served as controls.

Angiogenesis Assays

Co-Culture Angiogenesis Assay #1: EC/SMC contact model—SMCs were fluorescently labeled green with PKH-67 (10 μ M) and ECs were fluorescently labeled red with PKH-26 (10 μ M) per manufacturer instructions. PKH is a cell membrane label which

incorporates into the plasma membrane of cells and is divided equally into subsequent progeny cells.(Fox, Kouris et al. 1999) An aggregating solution consisting of angiogenesis assay media (M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (0.25 µg/mL), aprotinin (100 KIU/mL), heparan sulfate (5U/mL)), 2250 green fluorescently labeled SMCs, and a 20% Methocel solution (Uriel, Brey et al. 2006) was added into each well of a round bottom 96-well plate and incubated at 37°C for 24 hours until cell aggregates formed. After 24 hours, 350 red fluorescently labeled ECs were added to each well and allowed to seed the surface of the SMC aggregate. These cell numbers were based on preliminary experiments which optimized EC seeding to form a co-culture aggregate with an SMC core and as close to an EC monolayer as possible. In experiments which tested the effects of inhibiting SMC proliferation on EC matrix invasion, ECs were seeded onto aggregates of SMCs which were either treated or untreated with MMC as described above. To ensure that only SMCs were treated with MMC, the SMCs were washed with PBS 3 times prior to use in co-culture assays. The co-culture aggregates of ECs and SMCs were then embedded between two layers of fibrin that were polymerized such that the aggregates were completely surrounded by the hydrogel. Fibrin hydrogels were constructed by preparing a fibrinogen solution consisting of fibrinogen (2.5 mg/mL), M199, and heparin (5U/mL). The solution was polymerized by the addition of thrombin (0.32U/mL). Once completely polymerized, the disks were cultured in angiogenesis assay media (M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (0.25 µg/mL), aprotinin (100 KIU/mL), heparan sulfate (5U/mL)) at 37°C (Figure 1). After a period of time, SMCs and ECs invade from the central aggregate into the surrounding hydrogel as described in previous publications.(Figure 2)(Ucuzian, Brewster et al., Xue and Greisler 2002) Aggregates of 2500 fluorescently labeled ECs and no SMCs served as the control group.

Digital images were taken daily for up to 19 days at a magnification of 4x and quantified using a Zeiss Axiovert 200M microscope (Carl Zeiss, Inc, Oberkochen, Germany) and Axiovision software. The digital photographs were aligned with a grid evenly divided in 36 radial intervals using Adobe Photoshop for quantification using both TRITC and FITC filters. For each assay, the average length of sprouts (ALS) was defined as the average of the furthest distance of invasion from the central aggregate on each grid line. A secondary data point quantifying the density of sprout formation (DSF) was also measured by calculating the number of grid lines with crossing sprouts/36 total grid lines (Figure 2). The presence of cellular debris and discontinuity of previously continuous chains of invading cells is indicative of cell death.(Uriel, Brey et al. 2006) For this reason, sprouting was quantified by measuring only out to the final point of a continuous chain of cells. Notably, invasion into an extracellular matrix is a key component of angiogenesis. Thus, for the purposes of these studies, the quantification of matrix invasion by EC “sprouts” was a proxy endpoint for the angiogenic response of ECs. We have identified lumens by morphologic analysis in the studies described below. In addition, our previously published and unpublished data have demonstrated that ECs invading into fibrin hydrogels form lumens, while SMCs do not. (Brewster, Ucuzian et al., Xue and Greisler 2002)

Each individual experiment was analyzed by Student’s T-test with a α of 0.05 with statistically consistent results. The experiment was performed three times with consistent results with sample sizes of 4–6 per group per experiment. Data from a representative experiment is presented in the results.

Co-Culture Angiogenesis Assay #2: Multiple Co-culture Aggregate Assay—

Multiple co-culture aggregates were embedded within the same hydrogel to test for directional invasion towards the other aggregates. Co-culture aggregates were created as described above. Multiple aggregates were embedded approximately 300–500 µm apart between two layers of fibrin gels in the wells of flat-bottom 96 well plates and cultured in

angiogenesis assay media containing at 37°C. Once again, digital images were taken at a magnification of 4x in order to visually demonstrate the direction of radial invasion by co-culture sprouts. The experiment was performed 5 times, and representative images are reported.

Co-Culture Assay #3: ECs aggregates surrounded by SMCs embedded within the hydrogel—To test the effects of SMCs on angiogenesis in the absence of early EC/SMC contact, co-culture assay #3 was developed. ECs were red fluorescently labeled with PKH-26 (10 μ M) and suspended in an aggregating solution (10,000 cells/well) consisting of angiogenesis assay media (M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (0.25 μ g/mL), aprotinin (100 KIU/mL), heparan sulfate (5U/mL)), and a 20% Methocel solution and incubated at 37°C for 24 hours until cell aggregates formed. SMCs were fluorescently labeled green with PKH-67 (10 μ M) per manufacturer instructions. Fibrin hydrogels were constructed as described above with green fluorescently labeled SMCs (400,000 cells/mL) homogenously polymerized within the fibrin hydrogel. ECs were embedded between two layers of fibrin gels in the wells of flat-bottom 24 well plates such that co-culture assays consisted of pure EC aggregates surrounded entirely by a fibrin matrix containing SMCs. In these experiments with higher concentrations of SMCs, the hydrogels were polymerized onto nylon mesh rings to minimize the impact of SMC-mediated gel contraction. EC aggregates embedded within hydrogels that did not contain SMCs served as controls. Angiogenesis assay media was added to the top of the hydrogels containing the cells, and the assays were incubated at 37°C. After a period of time, ECs invade from the central aggregate into the surrounding hydrogel. Digital images were taken daily for up to 13–17 days at a magnification of 4x using a Zeiss Axiovert 200M microscope (Carl Zeiss, Inc, Oberkochen, Germany) and the ALS and DSF for each assay was quantified as described above. (Figure 2) Data from three individual experiments with sample sizes of 3–4 per group per experiment were pooled and presented $\pm$ SEM (total n=10–12 per group). The results of individual experiments demonstrated consistent results.

Histology—At 13 days, representative hydrogels of co-culture model #3 were processed for cross-sectional morphologic analysis with transmission electron microscopy. The assay disks were fixed with a 1% glutaraldehyde/4% paraformaldehyde solution, postfixed in 1% osmium tetroxide, dehydrated in a graded series of acetone, and embedded in epoxy resin. Ultrathin sections were cut, collected on copper grids, and stained with uranyl acetate and lead citrate. The sections were viewed on a transmission electron microscope (H-600; Hitachi, Tokyo, Japan) at 75 kV.

Statistical Analysis

We used analysis of variance with Tukey posttests or Student's T-tests using SigmaStat (Systat Software Inc, San Jose, CA) at a α of 0.05 to determine statistical significance.

RESULTS

Co-Culture Angiogenesis Assay #1: EC/SMC contact model

The Effects of SMCs on EC sprouting in fibrin hydrogels—The modulation of the angiogenic behavior of ECs by direct contact with SMCs was tested utilizing a contact co-culture angiogenesis assay modified from the methods of Korff and Augustin. {Korff, 2001 #1257}

ECs that were co-cultured with SMCs demonstrated increased average length of sprouting compared to ECs that were cultured alone at all time points (Figures 3 and 4). At Day 7 the ALS of ECs in co-culture with SMCs was 205 $\pm$ 32 μ m versus 103 $\pm$ 31 μ m of ECs

cultured alone ($p=.08$). At Day 19, the ALS of ECs in co-culture was $327 \pm 58 \mu\text{m}$ versus $70 \pm 11 \mu\text{m}$ of ECs cultured alone ($p=.01$).

The DSF of co-cultured ECs was also significantly greater than that of ECs not in co-culture at Day 13 and Day 19 ($p=.014$ and $.007$, respectively).

Sprouting ECs and SMCs were found to co-invade into the extracellular matrix in close proximity to one another. (Figure 5) At Day 7, the average length of EC sprouts was $205 \pm 32 \mu\text{m}$ and the average length of SMC sprouts was $229 \pm 19 \mu\text{m}$ ($p=0.54$). Similarly, the average length of sprouts of ECs and SMCs did not significantly differ at Day 13 ($378 \pm 55 \mu\text{m}$ for ECs versus $564 \pm 79 \mu\text{m}$ for SMCs; $p=.23$). At Day 19, EC sprout lengths were significantly less than SMC sprout lengths ($327 \pm 58 \mu\text{m}$ versus $551 \pm 39 \mu\text{m}$; $p=.03$).

The effects of MMC-treated SMCs on EC sprouting in fibrin hydrogels—To quantify the effects of inhibiting SMC proliferation and matrix invasion on the angiogenic behavior of ECs, ECs were seeded onto aggregates of SMCs that were either treated or untreated with MMC. MMC treatment had the effect of both inhibiting SMC proliferation and attenuating SMC matrix invasion as demonstrated in previously data.(Ucuzian, Brewster et al.)

After 7 days, ECs in co-culture with SMCs treated with MMC demonstrated significantly attenuated sprouting compared to ECs co-cultured with SMCs that were untreated with MMC ($82 \pm 14 \mu\text{m}$ versus $205 \pm 32 \mu\text{m}$; $p < .05$) (Figure 6).

Co-Culture Angiogenesis Assay #2: Multiple Co-culture Aggregate Assay

Directional Invasion from Multiple Co-Culture Aggregates—Multiple co-culture aggregates were embedded within individual fibrin hydrogels (Figure 7). Digital images were taken which included the embedded aggregates and the sprouts radiating from the aggregates. Sprouts arising from aggregates embedded alone within a hydrogel demonstrated random, non-directional sprouting by visual estimation (Figure 7, left panel). In contrast, sprouts from aggregates cultured with multiple other aggregates within the same hydrogel qualitatively demonstrated directional invasion towards sprouts from nearby aggregates within the same hydrogel (Figure 7, right panels). Assays were performed 5 times with representative images demonstrated in the figure.

Co-Culture Assay #3: Angiogenesis in ECs surrounded by, but not in contact with, SMCs

SMCs Effects on Angiogenesis in Absence of early SMC/EC contact—To determine the effects of SMCs in the absence of early SMC contact, angiogenesis from EC aggregates embedded within fibrin hydrogels that contained SMCs (400,000 cells/mL) were compared to angiogenesis from EC aggregates embedded in fibrin hydrogels which did not contain SMCs (Figure 8). The assays were cultured in angiogenesis assay media for 17 days. By Day 3, the ALS of ECs cultured in the presence of SMCs was significantly greater than those cultured in the absence of SMCs ($320 \pm 21 \mu\text{m}$ versus $187 \pm 16 \mu\text{m}$; $p < .005$). This significant difference persisted throughout the course of the entire experiment.

Angiogenic networks in co-culture continued to grow throughout the course of the experiment with the peak average length of co-cultured sprouts being $462 \pm 13 \mu\text{m}$ at Day 17. The ALS of ECs cultured in the absence of SMCs at Day 17 was $202 \pm 27 \mu\text{m}$, with a peak ALS of $233 \pm 8 \mu\text{m}$ at Day 13.

Similarly, ECs in co-culture formed more robust sprout networks, with the density of sprout formation (DSF) of co-cultured ECs being $42 \pm 8\%$ versus $12 \pm 2\%$ in ECs cultured in the absence of SMCs at Day 3 ($p=.01$). At Day 17, the DSF of co-cultured ECs was $69 \pm$

25% versus 7 $\pm$ 3% in ECs not in co-culture ($p = .02$). The peak DSF in co-cultured ECs was 76 $\pm$ 7% at Day 13 versus 31 $\pm$ 4% at Day 9 in ECs not in co-culture.

Morphology—Fluorescent microscopic evaluation of angiogenic sprouts demonstrated endothelial cell sprouts radiating from the central aggregate into the hydrogel matrix. Fluorescently labeled SMCs were found to closely associate with both the EC aggregate as well as individual endothelial cell sprouts (Figure 9). TEM images demonstrated cross-sections of luminal structures comprised of 2–3 nucleated cells surrounded by extracellular matrix and closely associated with surrounding cells on the abluminal surface of the structure (Figure 9).

DISCUSSION

Perivascular cells such as pericytes, SMCs, and adventitial fibroblasts, are associated with the endothelium of capillaries and are known to be involved in the later stages of angiogenesis during vessel maturation and stabilization. Direct contact via gap junctions, adhesion plaques, and adherens junctions, as well as indirect paracrine mechanisms between endothelial cells (ECs) and pericytes, allow for significant interactions which impact EC differentiation, migration, proliferation, and quiescence. (Gerhardt, Wolburg et al. 2000, Rucker, Wynder et al. 2000, Gerhardt and Betsholtz 2003, Tillet, Vittet et al. 2005) We demonstrate the potentially critical role of mesenchymally-derived cells such as SMCs in augmenting angiogenic activity of ECs. In our studies, we demonstrate that SMCs have the effect of augmenting EC invasion into a 3-D extracellular matrix, as ECs in contact with SMCs in a co-culture model of angiogenesis demonstrate greater lengths of invasion into a fibrin hydrogel compared with ECs cultured in the absence of SMCs. Notably, the presence of cell-cell contact between SMCs and ECs, was not sufficient in inhibiting the pro-angiogenic effects of SMCs during the time-course of these studies. We showed that even in the presence of SMC-EC contact, SMCs appeared to promote the invasion of ECs into the fibrin matrix, with ECs appearing to co-invade in close proximity to SMCs, with ECs ultimately lagging SMCs in matrix invasion. This suggests a direct function of SMCs in promoting EC matrix invasion, a key component in the angiogenic behavior of ECs. Indeed, the inhibition of SMC proliferation and invasion alone by treatment with MMC, a known anti-proliferative drug, inhibited both SMC and EC matrix invasion. While MMC may affect other SMC behavior other than proliferation, these data suggest that proliferating, or at least synthetically active, SMCs provide a pro-angiogenic stimulus for ECs to invade into the extracellular matrix.

We did not investigate the proliferative capacity of ECs which were co-cultured with MMC treated and untreated SMCs. However, as proliferation has been shown to be a critical component of matrix invasion in our previously published data (Ucuzian, Brewster et al.), we speculate that the augmentation of EC proliferation by SMCs may be one component of the pro-angiogenic response to co-culture with SMCs. It would be interesting to determine the proliferative capacity of ECs in co-culture with both synthetically active and inactive SMCs and compare it to the proliferative capacity of ECs cultured in the absence of SMCs.

The results of these studies appear contradictory to the generally held belief that once incorporated into capillaries, pericytes, which are derived by mesenchymal cells such as SMCs and fibroblasts, among other cells, mediate the quiescence of ECs during angiogenesis. *In vitro*, ECs in direct contact with pericytes have lower levels mitosis compared to ECs cultured alone, a process which may require Rho GTPase activity in pericytes. (Orlidge and D'Amore 1987, Hirschi, Rohovsky et al. 1999, Kutcher, Kolyada et al. 2007) Similarly, ECs in close contact with SMCs in a spherical aggregate of SMCs in collagen gels demonstrated decreased responsiveness to VEGF (in the absence of Ang-2),

increased inter-endothelial junctions, decreased PDGF-B growth factor chain expression, and reduced apoptosis. (Korff, Kimmina et al. 2001) ECs in co-culture with 10T1/2 cells also display a significantly higher resistance to the permeability of a tracer molecule biotin-dextran, which was associated with tightening of EC-EC tight junctions and with the localization of tight junction proteins plakoglobin, ZO-1, ZO-2, and occludin. (Kurzen, Manns et al. 2002)

The observations in our experiments, however, clearly show that SMCs in close contact with ECs can promote matrix invasion of ECs central to angiogenic processes. Thus, we conclude that the presence of cell-cell contact alone is not sufficient in promoting EC quiescence within the examined time course in this model. Factors such as the components and structure of the extracellular matrix and culture media, EC and pericyte differentiation, the time course of pericyte-mediated effects (early vs. late in angiogenesis) and specific paracrine cytokines and growth factors released by either ECs or SMCs all likely contribute to cell behavior in co-culture systems.

We hypothesize that the release of pro-angiogenic growth factors in the early timeframes of our experiments likely account for the observed pro-angiogenic effect of SMCs. Supportive of this hypothesis, when SMC/EC co-culture aggregates were cultured within the same fibrin hydrogel, sprouts radiated preferentially towards sprouts radiating from adjacent co-culture aggregates. The preferential directionality is clearly evident between aggregates located within a maximal distance from each other, strongly suggesting inter-aggregate crosstalk. We suggest that the cellular aggregate produces chemokines and/or mitogens that are secreted, accounting for this effect. Furthermore, we demonstrated that SMCs surrounding, but in minimal contact with ECs, promoted angiogenic induction in comparison to ECs which were cultured in the absence of SMCs. We believe this further strengthens our hypothesis that SMCs augment angiogenic induction likely through a paracrine mechanism. Demonstration of incorporation of SMCs into the capillary networks by the 7-day time-point did not directly correlate with attenuated matrix invasion by ECs, further suggesting that SMC/EC contact is not sufficient in promoting EC quiescence.

We have not identified the factors contributing to these effects to date, though it is likely accounted for by a combination of secreted growth factors as well as extracellular matrix degradation products as a result of fibrinolysis by SMCs. We plan a series of experiments addressing the mechanisms of this crosstalk, and the physical properties of the matrix and aggregate distribution influencing this phenomenon. It is conceivable that the identity of the factors released by SMCs prior to significant incorporation into microvasculatures, differ from those released later in angiogenesis or after pericyte incorporation. This could be an explanation for the apparent pro-angiogenic effects of SMCs early during our co-culture assays, and the apparent contradiction to the above-described studies that demonstrated the stabilizing effects of pericytes on neo-capillary formation during the latter phases of angiogenesis.

Our data does suggest that SMCs do have the effect of promoting the persistence of EC sprouts. EC sprouts in co-culture with SMCs in this model demonstrate delayed regression compared with ECs sprouts cultured alone. Thus, the greater length and robustness of EC sprout networks in co-culture compared to ECs cultured alone appear to be a function of both the promotion by SMCs of both early EC invasion and later EC persistence.

Clinically, the simultaneous targeting of pericytes and ECs by pro- or anti-PDGF and VEGF therapy has been investigated in tissue engineering and oncologic research. Combination therapy has been shown in some studies to be more effective in disrupting tumor vascularity than either agent alone and, as such, combinatorial antitumor therapy is under investigation.

(Bergers, Song et al. 2003, Potapova, Laird et al. 2006, Hasumi, Klosowska-Wardega et al. 2007, Timke, Zieher et al. 2008) It is clear by these and other studies that more precise and effective strategies in modulating angiogenesis will necessitate the consideration of the effects of supporting pericytes and organ-derived mesenchymal stromal cells on newly forming capillary networks.

In sum, we believe we have demonstrated a novel method of promoting angiogenic behavior by ECs. Our data demonstrates both proof of concept that SMCs stimulate angiogenic induction as well as capillary persistence. This is likely mediated by a paracrine effect that is not necessarily inhibited by contact between SMCs and ECs, and appears to rely, at least in part, on SMC proliferation. A deeper understanding of the mechanisms responsible for the cross talk between ECs and SMCs and other pericytes will likely allow for more precise control of both pro and anti-angiogenic behavior for a broad array of applications. Further, beyond providing a model for studying hypothetical interactions between pericytes and ECs during angiogenesis, we believe our 3-D, hydrogel-based, co-culture models of angiogenesis, provide very practical tissue engineering applications for the bioengineering of microvascular structures that resemble native capillaries.

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Highlights

- SMCs co-cultured in contact with ECs increase EC invasion in a 3-D fibrin hydrogel
- SMC proliferation and matrix invasion is key in increasing EC invasion into fibrin
- ECs surrounded by, but not directly in contact with, SMCs within fibrin hydrogels form luminal sprouts, with SMCs incorporating on the abluminal surface, and demonstrate more robust angiogenic networks compared to ECs cultured alone
- Directed invasion between co-culture cell aggregates suggests a paracrine mechanism for the augmentation of angiogenic induction by SMCs

Figure 1. Co-culture assay #1. Left) Fluorescently green labeled SMC aggregate. Middle) Fluorescently labeled red ECs coating SMC aggregate. Right) Merged image of red ECs covering the surface of the green labeled SMC aggregate. Mag.10x. Bar 100 μ m.

Figure 2. Quantification of angiogenesis. Mag 4x. ALS=Average length of sprouts; DSF=Density of sprout formation; L=Length; R=Radius of EC pellet.

Figure 3.
EC sprouts in the presence and absence of SMCs. Only EC sprouts are shown for simplification.(Mag. 4x) Bar 200 μ m.

Figure 4.

Quantification of EC sprouting in co-culture angiogenesis assay. ECs were cultured in the presence and absence of SMCs in direct contact with SMCs and the average length of sprouts (left) and density of sprout formation was quantified (right). N = 5; * $p < 0.05$.

Figure 5.

Invasion of ECs and SMCs into fibrin from co-culture aggregates. Left) ECs (red) co-invade with SMCs (green) into fibrin hydrogels (Mag. 10x). Bar 200 μm. Right) Average length of EC and SMC sprouts from a co-culture aggregate. N = 5; * $p < 0.05$.

Figure 6.
 Inhibiting SMC proliferation with MMC inhibits both SMC and EC matrix invasion into fibrin hydrogels in the co-culture angiogenesis model. N = 5; * $p < 0.05$.

Figure 7.

Directional invasion of co-culture sprouts. (Left) Sprouts from individual co-culture aggregates demonstrate random invasion into the hydrogel. (Middle and right panels) Sprouts from co-culture aggregates demonstrate directional invasion towards other co-culture aggregates embedded within the same hydrogels. The two right panels demonstrate two separate hydrogels, the middle panel has two aggregates embedded within the hydrogel, the right panel has five (three seen). Asterisks represent location of the two aggregates embedded within the hydrogel which were out of the field of view. Magnification, 4x. Bar 200 μm .

Figure 8.

Top) Quantification of angiogenesis in the presence and absence of SMCs (400,000 SMCs/mL). Bottom) Angiogenesis in the presence and absence of SMCs (400,000 SMCs/mL). Only ECs shown for simplification (Mag. 4x). n = 10-12; * p < 0.05. Bar 200 μm.

Figure 9.

Top) Cross sectional morphology of co-culture sprouts by transmission electron microscopy after 7 days of co-culture. Selected images demonstrate EC lined luminal structures with SMCs apposed to the abluminal surface of the capillary. The sprouts and associated cells were completely surrounded by the fibrin extracellular matrix. Debris was present within the lumen of the sprouts in each of the sections. Bar 1 μm . ECM = extracellular matrix; L = Lumen; Arrows = ECs; * = SMCs. Bottom) Sprouting from co-culture assay at 7 days. Left) Fluorescently labeled green SMCs are homogeneously suspended within the fibrin hydrogel. Middle) Fluorescently red labeled ECs are seen sprouting from a central aggregate of ECs into the matrix. Right) Merged image of ECs sprouting into the ECM containing SMCs which associate with EC sprouts. Mag 10x. (Lower image) Digital zoom of merged image. Mag 20x. Bar 200 μm .