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Engineered Blood and Lymphatic Capillaries in 3-D VEGF-Fibrin-Collagen Matrices With Interstitial Flow

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ABSTRACT: In vitro endothelial cell organization into capillaries is a long standing challenge of tissue engineering. We recently showed the utility of low level interstitial flow in guiding the organization of endothelial cells through a 3-D fibrin matrix-containing covalently bound vascular endothelial growth factor (VEGF). Here this synergistic phenomenon was extended to explore the effects of matrix composition on in vitro capillary morphogenesis of human blood versus lymphatic endothelial cells (BECs and LECs). Different mixtures of fibrin and collagen were used in conjunction with constant concentrations of matrix-bound VEGF and slow interstitial flow over 10 days. Interestingly, the BECs and LECs each showed a distinct preference in terms of organization for matrix composition: LECs organized the most extensively in a fibrin-only matrix, while BEC organization was optimized in the compliant collagen-containing matrices. Furthermore, the BECs and LECs produced architecturally different structures; while BECs organized in thick, branched networks containing wide lumen, the LECs were elongated into slender, overlapping networks with fine lumen. These data demonstrate the importance of the 3-D matrix composition in facilitating and coordinating BEC and LEC capillary morphogenesis, which is important for in vitro vascularization of engineered tissues.

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Introduction

In vitro morphogenesis of organized cell structures constitutes a major common goal among tissue engineers, particularly endothelial capillary morphogenesis, since in vitro vascularization remains a serious barrier to transplanting engineered tissues and organs. The approach to reach this end includes the integration of biochemical, biomechanical and cellular components to form functional and cohesive tissue systems (Griffith and Swartz, 2006). "In vitro angiogenesis"—which broadly defines the transformation of endothelial cells into tube-like structures on 2-D coated plates or on/in 3-D gels—has been studied for decades (reviewed in Davis et al., 2002; Egginton and Gerritsen, 2003; Vailhe et al., 2001), yet the formation of branched, continuous, stable, and perfused endothelial cell networks continues to challenge basic scientists and tissue engineers (Jain et al., 2005). Approaches for artificial vascularization can be categorized as (A) actuation of vessel sprouting (angiogenesis) from surrounding tissue into an engineered tissue matrix post-implantation by releasing angiogenic growth factors in a diffusion-controlled (Richardson et al., 2001) or cell-controlled (Ehrbar et al., 2004; Zisch et al., 2003) manner, and (B) in vitro prevascularization of the matrix by seeding endothelial cells, potentially along with other cells for network stabilization, and angiogenic growth factors in 3-D gels (Black et al., 1998; Hudon et al., 2003; Levenberg et al., 2005) or on micropatterned surfaces (Dike et al., 1999), which may then become incorporated into the vasculature post-implantation (Tremblay et al., 2005). In

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these approaches, cells and biomolecular cues play key roles in morphogenesis.

Recent evidence has demonstrated the importance of low-level interstitial flow in coordinating the organization of endothelial cells in 3-D cultures. The first studies demonstrated that interstitial flow could induce both blood and lymphatic endothelial cell (BEC and LEC) organization into 3-D structures in collagen gels when stimulated with phorbol 12-myristate 13-acetate (Ng et al., 2004). Subsequent studies in fibrin gels explored how interstitial flow would affect cell organization under the influence of matrix-bound vascular endothelial growth factor (VEGF) (Helm et al., 2005), a potent inducer of angiogenesis and vascular permeability. In addition, this compound exists in multiple isoforms, including the predominant matrix-binding $VEGF_{165}$ and the soluble $VEGF_{121}$ (Ferrara et al., 2003). While $VEGF_{165}$ has been shown to be more effective in inducing morphologically relevant angiogenesis than $VEGF_{121}$ (Keyt et al., 1996), an engineered fibrin-binding variant of $VEGF_{121}$, $\alpha_2PI_{1\text{-}8}$-$VEGF_{121}$, was more effective in driving proliferation and capillary morphogenesis than soluble wild-type $VEGF_{121}$ (Ehrbar et al., 2005; Zisch et al., 2001). In addition, when applied as an implanted fibrin matrix in vivo, this matrix-binding variant of $VEGF_{121}$ produced patent capillary vessels as opposed to the leaky variety elicited by soluble $VEGF_{121}$ in the same system (Ehrbar et al., 2004).

We showed that interstitial flow dramatically synergizes with this VEGF fusion protein incorporated into fibrin to produce complex capillary morphogenesis from LECs or BECs seeded in a 3-D single-cell suspension (Helm et al., 2005). Moreover, computational studies of VEGF liberation from the matrix, diffusion, and convection suggested that the interstitial flow acts to bias and amplify the distribution of cell-liberated VEGF, creating directional transcellular protein gradients to which the cells could directionally sense and respond (Fleury et al., 2006). While this study introduced a novel mechanism for how interstitial flow could enhance and direct cell migration and cell-cell communication, and demonstrated, importantly, that biomechanical cues can act in synergy with growth factor cues to affect *organization* in morphogenesis, it had limited use for certain tissue engineering applications because the fibrin cultures were not sustainable over long periods of time (>6 days). As a temporary wound healing matrix, fibrin is highly cross-linked and not amenable to remodeling by the cells but rather, by design, readily biodegraded by the cells; collagen, a more stable matrix for in vitro purposes, is a physical gel that is merely entangled but not cross-linked.

Here we seek to further exploit the coordinated action of slow interstitial flow and matrix-bound VEGF to achieve blood and lymphatic capillary organization in vitro with more extensive and stable capillary networks. We altered the growth matrix itself by adding various amounts of collagen and changing the relative amounts of fibrin, which allowed us to extend the time period of observation to at least 10 days. As before, we saw drastic synergy between the biophysical (organizational) cue of flow and the biochemical (growth) cue of matrix-bound VEGF. Interestingly, we found that the two different endothelial cell populations presented by the blood and lymphatic lineages behaved differently in the matrices tested and organized preferentially in different matrices. In addition, the structures that formed differed in architecture: LECs organized into elongated structures with slender lumen, while BECs produced broad, complex branching structures-containing wide lumen. These data together highlight the importance of matrix selection in the study and exploitation of endothelial cell organization for both basic studies of endothelial cell biology as well as tissue engineering applications, and demonstrate a new extent to which organized and interconnected capillary networks can be achieved in vitro.

Materials and Methods

Cell Culture

Human dermal BECs and LECs, isolated from neonatal foreskins using the lymphatic-specific receptor LYVE-1, were a kind gift from Dr. Mihaela Skobe (Podgrabinska et al., 2002) and used at passages 7–9. These cells were expanded on gelatin-coated flasks in basal endothelial cell medium (Cambrex, Walkersville, MD) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1 μg/ml hydrocortisone acetate, 25 μg/ml dibutyril cAMP, and 1% penicillin, streptomycin, amphotericin mixture (all from Sigma, St. Louis, MO) in a 37°C/5% CO_2 incubator.

Matrix Formulations

A mutant of recombinant human $VEGF_{121}$, α_2-PI_{1-8}-$VEGF_{121}$, was created as described previously (Zisch et al., 2001). The resulting bi-domain fusion protein contained mature human recombinant $VEGF_{121}$ and the factor XIIIa substrate sequence from α_2-plasmin inhibitor, NQEQVSPL, at the amino terminus. AlexaFluor 647-modified human fibrinogen (Molecular Probes, Eugene, OR) was combined with unlabeled human fibrinogen (Sigma) at a mass ratio of 1:25 (labeled to unlabeled), producing the base fibrinogen solution used for all the matrices. Gel matrices were prepared from a mixture containing 100 ng/ml α_2-PI_{1-8}-$VEGF_{121}$, 2 U/ml human thrombin (Sigma), 2.5 mM $CaCl_2$ (Fisher, Fairlawn, NJ), and 2 U/ml human factor XIII (Baxter, Deerfield, IL) along with final concentrations of type I collagen and fibrinogen shown in Table I. LECs or BECs were suspended in this solution at 1.5×10^6 cells/ml and the mixture was pipetted into an interstitial flow culture chamber and allowed to

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Table I. Composition and stiffness of the four matrices tested.

	Matrix			
	A	B	C	D
Collagen (mg/ml)	1	1	0.5	—
Fibrinogen (mg/ml)	2	1	2.5	3
Stiffness (Pa)	366 ± 144	97 ± 22	411 ± 93	171 ± 17
Permeability ($cm^2 \times 10^{-9}$)	12.0 ± 4.0	12.0 ± 4.0	3.3 ± 0.5	1.4 ± 0.5

polymerize, as described previously (Ng and Swartz, 2003). The chambers were maintained, in cell culture medium, for 18–24 h under static conditions to allow for cell attachment.

Application of Interstitial Flow

The radial flow chamber was employed for a 3-D flow environment. It consisted of a cylindrical central core with a radius of 0.16 cm and an outer ring with a radius of 0.56 cm (both composed of porous polyethylene) and a vertical internal height of 0.16 cm (described in Ng et al., 2004). To initiate flow through the radial flow chamber cell culture medium, supplemented with 200 KIU/ml aprotinin (Sigma), was slowly pumped through the gel using a six-roller peristaltic pump (Cole Parmer, Vernon Hills, IL). This slow interstitial flow drove an average velocity of 4.5 μm/s through the gel (15.8 μm/s in the inlet and 2.4 μm/s at the outlet) with a measured pressure head of 2 cm of water. Static controls were maintained in the interstitial flow chambers with the same supplemented culture medium but without being connected to the pump. All experimental conditions were maintained for 10 days at 37°C and 5% CO_2 (2–4 experiments were performed for each matrix with each cell type).

Analysis

Media samples from each experiment were collected 24 h after the imposition of interstitial flow. Commercially available ELISA kits were used to estimate secreted amounts of human matrix metalloproteinase (MMP)-9 (Calbiochem, San Diego, CA) and MMP-3 (R&D Systems, Minneapolis, MN).

After the 10 days of culture, the gels were fixed in 4% paraformaldehyde and stained with 200 nM phalloidin conjugated with AlexaFluor488 fluorescein for f-actin and Sytox Orange for nuclei (both from Molecular Probes).

Image Acquisition and Quantification

Images of the matrix and endothelial cells were collected using a laser scanning confocal microscope (Leica LCS SP2). The collagen contained within the matrix was visualized using confocal reflectance, exploiting the reflective qualities of the collagen fibers under 488 nm laser illumination (Ng et al., 2005). Two sets of 3-D image stacks that spanned a depth of 40 μm and a length of 1.46 mm were collected in sequence from the inner core to the outer ring of each chamber. The volume occupied by multi-cellular organizations was measured and correlated for the resulting sets of stacks, based on pixel densities collected by Volocity (Improvision, Lexington, MA) using a size threshold that eliminated the counting of rounded cells. In addition, 2-D projections were created from these stacks to analyze other structure characteristics. The shape of the structures was assessed by measuring the projected area and perimeter of each structure (Volocity) and computing their ratio. A size threshold of 300 μm^3 was used to preclude the individual rounded cells. The extent of organization was also determined from the 2-D projections. Two highly organized regions, each spanning 40% of the radial distance, were used from each sample to manually count the number of cells involved in each structure.

Gel Stiffness Measurements

A Paar Physica MCR Rheometer (Anton Paar, Graz, Austria) was used to measure the viscoelastic properties of matrices created with the same formulations that were used in the flow experiments. The matrix constituents were mixed, as detailed above, and pipetted directly onto the stainless steel cone and plate of the rheometer which were maintained at 37°C in a humidified chamber. The cone measured 50 mm in diameter with a 1° angle. Measurements were collected over 15 s every 10 min for a total period of 2 h; the matrix was allowed to relax during the 10 min intervals. The rheometer was operated in oscillating mode at 1% strain and a frequency of 10 s^{-1}. The storage modulus G' was calculated by the MCR US200 software and was used to represent the stiffness of the tested matrices. Data shown in Table I indicate the mean ± SEM.

Gel Permeability Measurements

The hydraulic permeabilities of each gel formulation were assessed without cells. An in-line manometer was included between the feed pump and flow chamber inlet. Cell culture medium was perfused through the flow chamber while housed in a 5% CO_2, 37°C incubator. For each of four flow rates, the system was allowed to run for 1 h and the resulting pressure differential was measured. Darcy's law, $v = (-K/\mu)\nabla P$, was used to calculate the permeability K, where v is the average velocity, ∇P is the pressure gradient dP/dr, and μ is the fluid viscosity (measured as 0.7657 cP using an Ubbelohde viscometer at 37°C). Because of the radial flow geometry, this was rewritten as $Q = B(K/\mu)\Delta P$, where

ΔP = inlet pressure − outlet pressure, $B = 2\pi h/\ln(r_o/r_i)$ and h = chamber height, r_o = outer radius, and r_i = inner radius. In this way, the slope of the Q versus ΔP line was used to calculate K.

Statistics

Comparisons of the measurements among matrix formulations within each cell type (LEC or BEC) were conducted using the Kruskal–Wallis analysis of variance followed by Dunn's comparison. Comparisons between cell types for a given matrix were made using a two-tailed Student's t-test while the error bars shown correspond to mean ± SEM.

Results

With the exception of matrix B, the four matrix formulations had equal total protein concentrations but varied in terms of their relative amounts of fibrinogen and collagen according to Table I. Each matrix contained the same concentration of α_2-PI_{1-8}-$VEGF_{121}$ (100 ng/ml), thrombin (2 U/ml), $CaCl_2$ (2.5 mM), and factor XIII (2 U/ml). These differences in extracellular matrix (ECM) composition translated into a range of stiffness values, as indicated by the storage modulus, G', from 100 to 400 Pa; matrices A and C had similar high values, and matrices B and D had similar low values. Permeability values for these matrices (Table I) showed that matrices A and B (the ones with the most collagen) had the highest permeability and matrix D (with no collagen) the lowest.

Images of the fibrous gel structures were collected using confocal microscopy, allowing for their assessment in a hydrated state, and eliminating potential artifacts of analysis by electron microscopy (Pedersen and Swartz, 2005). A comparison of the architectures of each matrix (Fig. 1) demonstrate that fiber density increased with increasing fibrinogen concentration, consistent with earlier findings (Herbert et al., 1998; Ryan et al., 1999). The collagen present in matrices A–C appears as a fibrous network that interpenetrates within the fibrin network, producing a dense complex growth environment for the cells.

Interestingly, BECs and LECs each organized differently in the four different matrices (Fig. 2). Specifically, while BECs organized better in matrices with higher collagen concentrations (and higher compliance), LEC organization was best in the fibrin-only matrix (which was also a more compliant gel). After 10 days under interstitial flow, BECs formed extensively branched structures in matrices A and B, many of which spanned large distances (>400 μm), particularly in the case of matrix B. In matrices C and D, BECs failed to organize substantially. In contrast, LEC organization was observed in matrices B and D, but not in A or C. Additionally, no significant difference in cell organization was detected as a function of radial position (data not shown).

These differences were quantified in two ways: first, the volume occupied by the structures reflected the overall

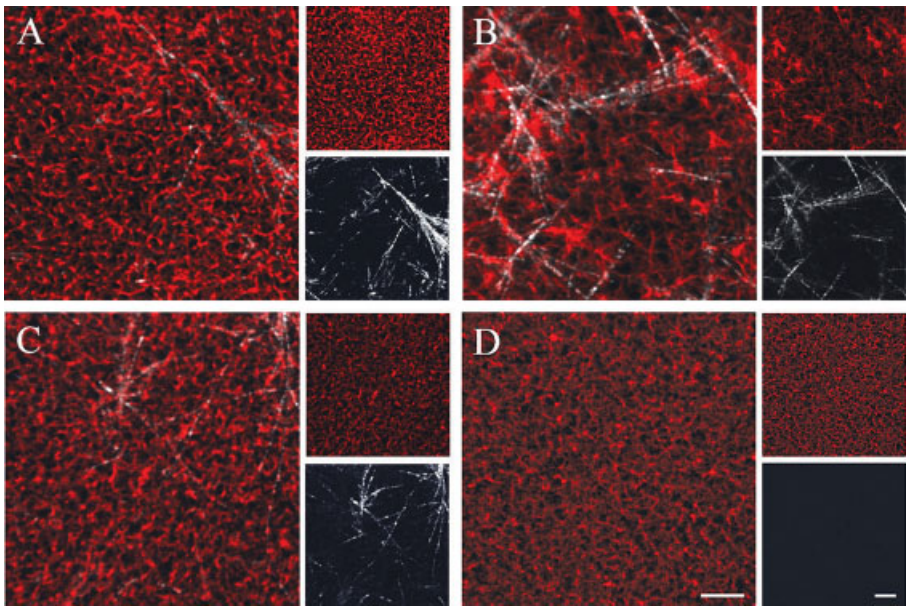

Figure 1. Structural architecture of the four matrices described in Table I. **Panels A–D** show confocal images of the fibrous structure in matrices A–D, respectively. The fluorescently labeled fibrinogen is indicated in red, while confocal reflectance shows the presence of collagen fibers in gray that are well dispersed within the predominant fibrinogen. The smaller individual red and white channels show each matrix constituent separately. Scale bar = 10 μm.

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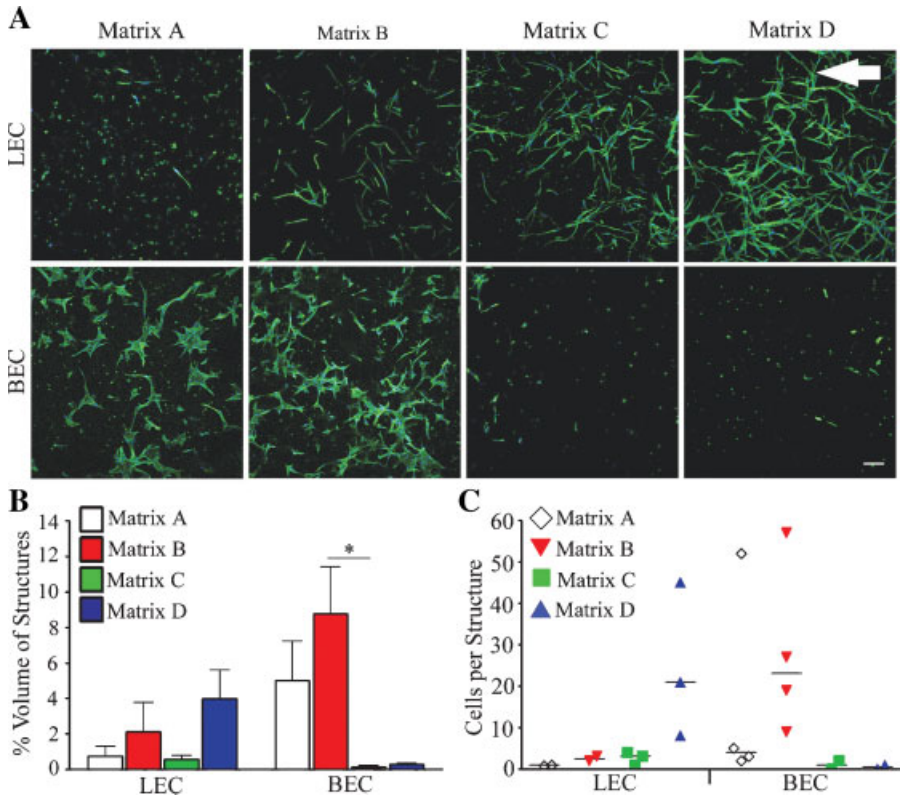

Figure 2. Organizational behavior of LECs and BECs in matrices A–D under interstitial fluid flow. **A**: 2-D projections of confocal stacks showing endothelial organization as indicated; Green = f-actin; blue = nuclei; scale bar = 80 μm. **B**: The total percent volume occupied by multi-cellular structures in each of the four matrices, which reflects the degree of organization (i.e., how many structures form), demonstrates that matrix B was optimal for BEC organization while matrix D was optimal for LEC organization. $^{*}P < 0.05$ using Kruskal–Wallis analysis with Dunn's post-test. **C**: Multi-cellular organizations were assessed for the average cell number per structure to quantify the extent of endothelial organization (i.e., how big the structures are). Lines on scatter plot indicate median values for each data set. This also showed that matrices B and D were optimal for BEC and LEC organization, respectively. In all matrices, interstitial fluid flow moved from right to left.

degree of organization (Fig. 2B); and second, the counts of cells per structure reflected the extent of organization (Fig. 2C). For BECs, matrices A and B provided the best environment for both degree and extent of organization (Fig. 2B); however, matrix B elicited a better overall response in both degree (Fig. 2B) and extent (Fig. 2C) of organization. BECs contained within matrices C or D failed to organize significantly. In contrast, the degree of LEC organization was best in matrices B and D (Fig. 2B), but only matrix D showed an enhanced extent of organization. In other words, although several matrices appeared to support LEC organization, only matrix D supported the formation of structures more than 2–3 cells in size (Fig. 2C). Neither cell type organized to any significant degree or extent in matrix C, which had the combination of low collagen concentration but high stiffness compared to the other matrices (note that matrix D had no collagen at all, but it was twice as compliant).

Aside from preferring different matrix compositions for optimal organization, the LEC and BEC structures that formed had markedly different morphologies. LEC structures were very slender and elongated, similar to that seen in previous, shorter duration studies (Helm et al., 2005), while BEC structures were broader with more densely packed cells. Closer examination of the LEC and BEC structures in higher magnification images revealed more detail about their architecture (Fig. 3). The BEC structures that formed were reminiscent of in vivo vascular endothelium with cells tightly packed and aligned along the edge of broad open lumen spaces (Fig. 3A–D). In vitro LEC structures also mimicked their in vivo architecture, with adjacent cells overlapping in the slender organizations they formed (Fig. 3E) and with slight lumen (Fig. 3F).

We attempted to quantify these differences in shape by compiling the ratio of each structure's area to its perimeter when projected into a 2-D image. In this way, long and slender structures would have a smaller ratio value than broad structures. When comparing the average of these ratios, we found that BEC structures had a significantly higher value than LEC structures, indicating that these structures were indeed broader than those produced by LECs (Fig. 3G).

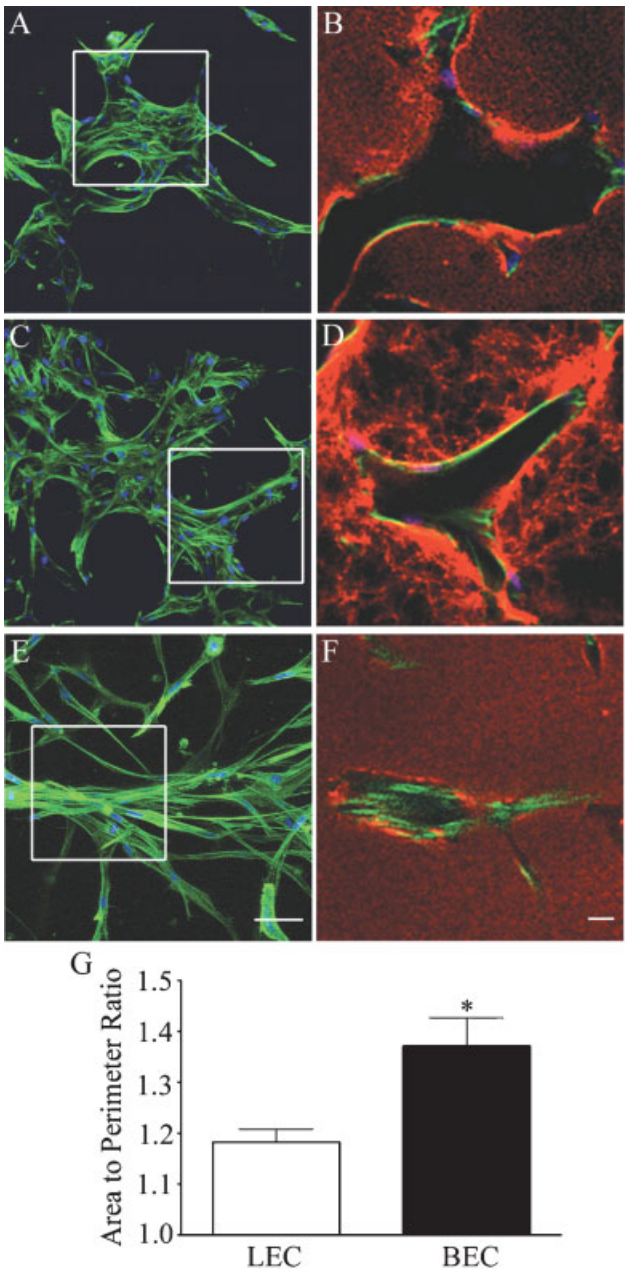

Figure 3. High magnification confocal images show that the BEC structures contained broad lumen while LEC structures produced very fine lumen in each of their most organizationally conducive matrices. **A**: BECs organized in broad, complex structures in matrix A. **B**: BECs in matrix A showing cell enclosed lumen void of matrix fibers, shown in red. **C**: BECs organized in broad complex structures in matrix B. **D**: Confocal images of BECs in matrix B showing enclosed lumen void of matrix fibers. **E**: LECs organize in long slender branched structures in matrix D. **F**: LECs in matrix D showing fine cell enclosed lumen void of fibers. Green = f-actin; blue = nuclei; red - = matrix fibers. **Panels A**, **C**, and **E** scale bar = 40 μm; **panels B**, **D**, and **F** scale bar = 10 μm. **G**: To characterize shape of structures that formed, the total structure area and perimeter were measured in 2-D projections of 3-D stacks. For each cell type in its optimal matrix formulation, the area-to-perimeter ratios were tabulated and averaged; $^{*}P < 0.05$ using a Student's *t*-test.

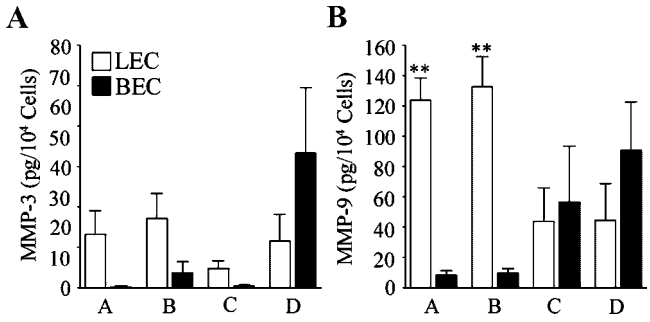

Figure 4. BECs produced significantly less MMP-9 then LECs in matrices A and B while MMP-3 expression was consistent across cell types and matrices. **A**: MMP-3 expression measured in flow chamber effluent medium. LECs indicated by white bars and BECs indicated by black bars. **B**: MMP-9 expression measured in flow chamber effluent media. $^{**}P < 0.005$ BEC versus LEC as determined by Student's *t*-test.

In order to determine whether the different matrices differentially affected the proteolytic activity of the embedded cells, we also measured expression level of relevant MMPs. When the expression of MMP-3 was assessed, little difference was seen across the LEC population when comparing the results of the different matrices (Fig. 4A). The BECs showed some differences (although not statistically significant)—namely that MMP-3 secretion was notably higher in matrix D, which was the only matrix with no collagen. However, this was unrelated to organization, since BECs neither organized in matrix D nor in matrix C, where MMP-3 secretion by BECs was low. MMP-9 secretion, however, was significantly and substantially different between LECs and BECs in matrices A and B (Fig. 4B); interestingly, these were the matrices with the most collagen, so the LEC response of MMP-9 secretion may reflect attempts by LECs to remodel those matrices with more collagen as compared to matrices C and D. In contrast, BECs secreted the most MMP-9 in matrices C and D, where they did not organize; again, this may reflect an attempt to remodel their surroundings to make them more optimal for cell organization.

Discussion

We show here that LECs and BECs organize into capillaries differentially when cultured in matrices containing varying amounts of collagen and fibrin, under the influence of both matrix-bound VEGF and interstitial flow. Specifically, LECs organized best in the fibrin-only matrix (and, interestingly, the one with the lowest hydraulic permeability), and formed long, slender, and overlapping structures, while BECs organized best in collagen-fibrin mixtures that were the most permeable to fluid movement and formed densely cellularized branched tubes. For both cell types, more compliant matrices were more conducive to capillary

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morphogenesis. These differences were not apparently due to differences in soluble proteases secreted by the cells or in the amounts of VEGF released from the matrix, implying that the cell interactions with the matrix constituents, as well as possibly in response to the resistance to fluid flow though the matrix, were largely responsible for the differences in organizational behavior seen.

The ECM serves multiple roles, acting as a structural support, a reservoir for biochemical signals, and as an actuator itself of both chemical and mechanical stimuli. Several cytokines such as VEGF, heparin, and basic fibroblast growth factor are known to be sequestered or stored in the ECM for use by cells in their presence (Fairbrother et al., 1998; Fannon et al., 2000). Furthermore, the constituents that comprise the matrix can themselves act to affect the cells it supports. As Feng et al. (1999) point out, the growth of endothelial cells in contact with one matrix constituent or another, namely collagen or fibrin, can drastically affect which integrins they produce and subsequently the signaling pathways that can be triggered. In addition, Jalali et al. (2001) demonstrated the need for integrin–ECM interactions in order for proper transmission of mechanotransduction signaling pathways in endothelial cells. Both studies highlight the role that the particular ECM elements play in communicating with their resident cells. Generally, the ECM plays a key role in the communication scheme of the cells within, whether they be assessing their surrounding environment and reacting accordingly or communicating with other nearby cells.

The matrices utilized in this study were patterned after the ECM matrix present during the wound healing response. Under these circumstances, fibrin and collagen serve as provisional matrices for the infiltration of inflammatory cells and reorganizing endothelial cells. The initial presence of fibrin (in the form of a clot) is replaced gradually, but over a brief time period, by the infiltration of collagen produced by invading fibroblasts (Clark et al., 1995). Thus, during this wound healing process both matrix elements are present at once, providing the support structure for regenerating vasculature. Consequently, the use of matrices with varying proportions of fibrin and collagen provide the endothelial cells with an environment reminiscent of what can occur in some in vivo situations.

Although the biomolecular environment has been demonstrated to play a key role in the numerous studies exploring the organization of endothelial cells in vitro, the matrix itself also serves a crucial role that should be considered (Pepper et al., 1996; van Hinsbergh et al., 2001). Here we see that the choice of matrix alone can determine whether endothelial cells will organize into complex capillary structures or remain as individually suspended cells in a gel, even when stimuli proven to yield organization are applied. As has been demonstrated by recent studies, interstitial flow is a strong inducer of endothelial cell organization in both fibrin and collagen gels, and synergizes with VEGF in driving capillary morphogenesis (Helm et al., 2005; Ng et al., 2004). Exploiting this phenomenon, while varying the constituents of the matrix, produced an interesting panel of results. In general, BECs and LECs organized best in matrices that were more compliant—matrices B and D, respectively. Previous work by Nehls and Herrmann (1996), as well as Urech et al. (2005) highlighted the morphogenic preference that both venous and arterial endothelial cells had for more compliant fibrin matrices. These softer matrices, when explored in vitro, not only allow for cell organization on the surface of fibrin gels but also allow for invasion and organization within its interior to a degree not seen in stiffer matrices (Nehls and Herrmann, 1996; Vailhe et al., 1998; van Hinsbergh et al., 2001).

The fibrin and collagen content of each matrix explored in this study differed in compliance and permeability, and facilitated cell–matrix interactions through integrins that elicit unique functions and cell signaling events (Boudreau and Jones, 1999). Certainly, the role of the matrix components as structural regulators of the cellular organization cannot be overlooked. Interestingly, while the overall protein content of the matrices differed in only one formulation, the calculated permeabilities were different across all four. These values generally corresponded with previously collected data (e.g., the permeability of a 3 mg/ml fibrin gel was on the order of 10^{-9} cm^2 (Sjoland, 2005)). In addition, the permeability values appeared to correlate with the overall collagen content of each matrix formula, where the matrices with the highest permeability, A and B, had the highest collagen content, and decreases in the collagen resulted in an increase in overall permeability. We found that BECs and LECs each favored different matrix constituents to elicit their greatest degree of organization. Matrix B, favored by BECs, contained an equal proportion of both collagen and fibrin and displayed the highest hydraulic permeability, while matrix D, the LEC-preferred matrix, contained only fibrin and displayed the lowest permeability. Thus, a combination of biophysical and molecular cues contribute to the differential organization of each cell type, and it is possible that LECs respond to a higher resistance to fluid flow as a functional driver. This is consistent with earlier findings that interstitial fluid flow was an important driver of lymphatic organization in regenerating mouse skin (Boardman and Swartz, 2003; Rutkowski et al., 2006).

These results are also interesting in light of wound healing events in vivo, where blood angiogenesis occurs within a few days, when the fibrin clot is still intact but collagen is also present (Singer and Clark, 1999). However, lymphangiogenesis begins many days later (Rutkowski et al., 2006), when the matrix has likely remodeled drastically. Since the overall stiffness values of these matrices were quite similar, the differences in cell behavior within them could also have been influenced by their differences in permeability as well as the nature of the matrix components themselves. The primary means by which cells interact with their surrounding ECM is through integrins which are specific to particular constituent molecules. Endothelial cells possess integrins,

such as $\alpha_v\beta_3$ and $\alpha_2\beta_1$, which are specific to fibrin and collagen, respectively (Albelda et al., 1989; Brooks et al., 1994; Herrick et al., 1999). In addition, the responsiveness of integrins to stimulation by VEGF can also result in added capillary morphogenesis (Chavakis et al., 2004; Hall and Hubbell, 2004). Here the demonstrated preference of LECs and BECs for different matrix constituents in which to organize may be attributed to the action of integrins and their potential interaction with other cell receptors.

Since the different matrix compositions have multiple differences in terms of properties that can affect cell organization—including mechanical properties, integrin ligand presentation, proteolytic sensitivity, cell migration strategy, and cytokine storage and presentation—it is difficult to speculate the reasons why the two cell types organized optimally in each of the specific matrices that they did. The interstitial fluid velocity used in the experiments, 4.5 μm/s, was higher—but within less than an order of magnitude—as that measured previously in vivo (Chary and Jain, 1989; Dafni et al., 2002). Furthermore, as has been demonstrated recently, interstitial flow through a cell-containing 3-D matrix has the ability to distort the pericellular distribution of cell-produced cytokines and proteases, which would affect both their autocrine and paracrine signaling and thus, presumably, alter their organizational behavior (Fleury et al., 2006; Helm et al., 2005).

A variety of proteases are expressed by endothelial cells and are involved in their migration and organization within the ECM (Haas, 2005). The LEC and BEC organizations we observed within the fibrin and fibrin-collagen blends was certainly proteolytically controlled, as seen in vivo in similar matrices (Cassell et al., 2002). Although we did not exhaustively explore the exact proteolytic signatures of the LECs and BECs during organization, we did assess two candidate MMPs that were likely to be involved in the organization process. The cell-produced proteases that are responsible for VEGF liberation from the matrix and, ultimately, cell organization may have caused the differences seen in LEC and BEC organization. Although direct measurement of the free (soluble) VEGF released showed no significant differences among all the conditions (data not shown), the presence of proteases in the organization phenomenon required further exploration. We examined MMP-3 and -9 because of their active roles in angiogenesis (Pepper, 2001) and their ability to degrade the utilized matrix components (Bini et al., 1999; Nguyen et al., 2001). However, the relative expression of these soluble MMPs did not lend insight into the mechanistic differences in cell behavior between the different matrices. We found that MMP-3 was secreted in similar levels by LECs across all matrices, but not in BECs, where (1) MMP-3 secretion was generally lower than that by LECs and (2) MMP-3 secretion was notably (but not significantly) higher in matrix D, which was the only matrix with no collagen. Thus, it is likely that MMP-3 release by BECs (or lack thereof) may be partly regulated by collagen binding. Furthermore, since BECs responded best to collagen-containing matrices while LECs responded best the fibrin-only matrix, it is interesting to note that BECs secreted the most MMP-9 in matrices with the least collagen (C and D, where BEC organization was poorest), while LECs secreted the most MMP-9 in matrices with the most collagen (A and B, where LEC organization was poorest). Thus, MMP-9 expression may indicate an attempt by the endothelial cells to remodel their ECM in order to make their environments more conducive to morphogenesis.

In summary, these data emphasize the importance of matrix selection in the success of in vitro vascularization of 3-D tissue constructs. More specifically, compliant matrices composed of a blend of collagen and fibrin or just fibrin alone are well suited for supporting blood and lymphatic capillary morphogenesis, respectively. The ultimate functionality of these structures has yet to be determined, but further analysis of their role in facilitating tissue oxygenation (Jain et al., 2005) would be beneficial to the ultimate use of the described technique in vascularizing engineered tissues. Whether governed by specific interactions with the matrix constituents through mechanical and/or chemical interactions or variations in the cell produced proteolytic environment, the data presented highlights behavioral differences between the lymphatic and blood endothelial cell populations. These variations in behavior must be considered when designing tissue engineered systems that will need blood and lymphatic vasculature to ultimately become properly integrated into its intended in vivo location.

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