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Original Article

The Effect of Hypoxia on *In Vitro* Prevascularization of a Thick Soft Tissue

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Prevascularizing an implantable tissue is one strategy to improve oxygen (O_2) transport throughout larger tissues upon implantation. This study examined the role of hypoxia both during (i.e., as a stimulus) and after (i.e., mimicking implant conditions) vascularization of an implantable tissue. Tissues consisted of microcarrier beads coated with human umbilical vein endothelial cells embedded in fibrin. The fibrin was covered with a monolayer of normal human lung fibroblasts (NHLFs), or exposed to conditioned media from NHLFs. Capillary networks developed at 20% or 1% O_2 tension for 8 days. In some experiments, tissues were supplemented with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, whereas in others the tissues prevascularized at 20% O_2 were transferred to 1% O_2 for 8 additional days. Maximal capillary formation occurred in media conditioned by NHLFs at 20% O_2, supplemented with VEGF (concentration >10 pM). Hypoxia (1% O_2) did not stimulate basic fibroblast growth factor production and decreased *in vitro* angiogenesis, despite an increase in endogenous VEGF production. Hypoxia also degraded a preformed capillary network within 4 days. Hence, strategies to prevascularize implantable tissues may not require the physical presence of stromal cells, but will likely require fibroblast-derived growth factors in addition to VEGF to maintain capillary growth.

Introduction

Tissue engineering has the potential to revolutionize the way clinicians treat damaged tissues. By developing techniques that combine living cells with natural and synthetic biomaterials, tissue engineers are creating new therapies that restore, maintain, or enhance functionality of damaged tissues.[1–3] Despite intense interest and effort, there have been few successfully engineered tissues appropriate for implantation. Successes have been limited to thin (<2 mm) avascular tissues, such as epidermis and nasal septum cartilage, where diffusion is adequate for oxygen (O_2) and nutrient delivery.[4–6] Although larger tissues have been attempted, developing thick tissues is limited by O_2's slow diffusion relative to its consumption.[1,2,7] This limitation is overcome in the body by capillary networks that deliver O_2 deep within tissues, insuring that O_2 never diffuses more than a few hundred microns.[8] Overcoming these diffusion limits, to create large viable engineered tissue constructs is one of the greatest challenges facing tissue engineers.[9]

There are several proposed methods to overcome this challenge, including (1) implanting avascular implants doped with angiogenic factors to stimulate rapid vessel growth,[10–16] (2) seeding polymer scaffolds with endothelial cells (ECs) and angiogenic factors before implantation,[17,18] and (3) prevascularization, generating vasculature within a tissue construct before implantation.[19–21] All of these methods show varying degrees of success; however, the first two methods require spontaneous vessel formation postimplantation. During this time, diffusion is the sole transport mechanism to support the cells near the periphery of the implant, while there is no mechanism to support cells deeper within the implant. Prevascularization potentially overcomes this limitation by preforming a capillary network *in vitro*, but relies on rapid inosculation with the host vasculature upon implantation to ensure cells throughout the implant are supported.

Angiogenesis is a complex biological process where new vessels are formed from existing vessels.[22–25] Under normal conditions the angiogenic switch[26] favors negative regulation, until activated by one or more stimulators, such as hypoxia. Local hypoxia (<1% O_2) occurs when tissue damage reduces blood flow. Interstitial cells sense this change in O_2 tension and activate the angiogenic switch by secreting proangiogenic factors. Although numerous factors can influence angiogenesis, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the most extensively studied and well understood, and clearly stimulate vascularization.[25–28]

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A variety of cells respond to hypoxia by upregulating VEGF production and secretion. Secreted VEGF binds its cell surface receptors (cognate receptor tyrosine kinase VEGFR1 and VEGFR2) on the surface of ECs triggering vascular permeability, EC proliferation, and migration. bFGF is a strong mitogen for a variety of cells, including ECs, and is typically bound to the extracellular matrix. Pepper *et al.*[29] showed that VEGF and bFGF given in combination increased *in vitro* angiogenic activity of ECs more than each growth factor alone. More recently, Arkudas *et al*. demonstrated that fibrin gels loaded with VEGF and bFGF stimulated vessel growth in an arteriovenous loop model.[30] Numerous studies corroborate the synergistic effect VEGF and bFGF on angiogenesis.[29–33]

A macroscale engineering approach, where individual parameters affecting angiogenesis are manipulated and the overall outcome of these changes are observed, can facilitate the development of a thick prevascularized tissue. In an earlier report, we identified the maximal diffusion limits ($\sim$1.8 mm) of nutrients and soluble fibroblast-derived mediators that support stable capillary formation *in vitro*,[34] and also demonstrated that these vessels not only become functional upon implantation,[35] but also lead to earlier perfusion of blood, larger blood vessels, enhanced cell proliferation, and enhanced deposition of extracellular matrix.[36] Even though tissues up to 7.2 mm thick supported capillary formation, the O_2 tension within the tissue *in vitro* remained relatively constant and high (>125 mmHg).[34] Importantly, the presence of fibroblasts and exogenously added VEGF and bFGF were required for capillary formation in this model.

It is well established that hypoxia is a stimulator of angiogenesis through a number of mechanisms (mainly through hypoxia inducible factor-1 [HIF-1] stabilization leading to VEGF upregulation). Therefore, hypoxia is a critical parameter that can be controlled in an effort to improve vessel formation *in vitro*. Further, the wound bed where a prevascularized tissue is implanted is likely to be hypoxic (<19 mmHg of O_2).[37,38] The response of a preformed capillary network to a step change in O_2 tension remains unknown. The objectives of the present study are two-fold: (1) to determine the effect of hypoxia on vessel formation and growth factor production in an *in vitro* model of angiogenesis and (2) to identify how the tissue construct will behave in response to a step change in O_2 similar to that which will occur upon implantation into a hypoxic wound bed of the host animal.

Materials and Methods

Cell culture

Human umbilical cord vein endothelial cells (HUVECs) were isolated from freshly harvested umbilical cords. Collagenase type IA (Sigma, St. Louis, MO) digestion (1 mg/mL) was performed at 37°C for 15 min to release ECs from the vessel walls. Vessel walls were rinsed with Hank's balanced salt solution (Lonza, Basel, Switzerland), and the wash collected. The wash was centrifuged at 1200 rpm for 5 min. The pellet was re-suspended in endothelial growth media (EGM-2; Lonza) without VEGF and plated in collagen-treated culture flasks (Fisher Scientific, Pittsburgh, PA). Upon reaching 80% confluency, HUVECs were harvested with trypsin/EDTA treatment (Clonetics, San Diego, CA). Normal human lung fibroblasts (NHLFs; Lonza) were cultured in fibroblast growth medium (FGM-2; Lonza). Medium was changed every 48 h until the NHLFs reached 90% confluency, and the cells were harvested with trypsin/EDTA. HUVECs were passaged a maximum of three times, while NHLFs were used up to passage 10.

Assembly of vascularized tissue construct

Cytodex™ three microcarrier beads (Sigma) were sterilized and prepared for seeding with a series of washes in EGM-2 modified without VEGF. HUVECs (passage 3) were added (seeding density 4×10^6) to an inverted T25 culture flask containing 5 mL of EGM-2 without VEGF and approximately 10,000 Cytodex beads. The inverted flask was then incubated at 37°C with 5% carbon dioxide (CO_2) atmosphere. To facilitate bead coating, the flask was shaken gently for 1 min every 20 min. After 4 h, the microcarrier beads were adequately coated with HUVECs. The coated beads were transferred to a fresh T25 flask, an additional 5 mL of growth medium is added, and incubated in the standard cell culture position for 24 h allowing any suspended cells to attach to the bottom of the flask. A fibrin solution (2.5 mg fibrinogen (Sigma)/mL EGM-2 modified without fetal bovine serum) was prepared and sterile filtered. Before construct assembly, the EC-coated beads (200–250 beads/0.5 mL fibrin solution) and 2% fetal bovine serum were added to the fibrin solution. In each well of a 24-well plate (Fisher Scientific), 10 μL of a thrombin solution (50 U/mL) and 500 μL of the fibrin-bead solution were added. The construct was allowed to stand for 5 min, while the beads settle to the bottom of the well by gravity, before incubating for 20 min at 37°C and 5% CO_2. During incubation, the fibrin cross links (gels). The beads were seeded at a relatively low density to maximize the distance between neighboring beads and minimize clumping. NHLFs (25,000 cells/well) were plated on top of the fibrin gel and incubated for 1 h. The NHLF medium was then removed, and the appropriate growth medium formulation applied to each well. Growth medium was changed every 48 h for the duration of the 8-day experiments.

Hypoxia challenge

Fibrin tissue constructs (gels) were assembled as described above. Completed constructs were assigned to either control or 1% O_2 groups. Control tissues were maintained with room air ($\sim$20% O_2). Similarly, tissues assigned to 1% O_2 were maintained in a different incubator that limited the O_2 concentration (balance nitrogen) to 1%. Our previous work[34] demonstrated that the O_2 tension within the tissue construct is approximately equivalent to the incubator air with minimal spatial gradients. All of the conditions were maintained with 5% CO_2 and 37°C. Within each group (12 gels), 6 gels were maintained with either complete EGM-2 (i.e., with growth factors, +GF, VEGF, and bFGF) or EGM-2 formulated without −GF, VEGF, and bFGF, resulting in a 24-gel experiment with the following conditions: control +GF, control −GF, 1% +GF, and 1% −GF where control refers to 20% O_2 or room air. VEGF and bFGF were added in combination because earlier reports demonstrate the synergistic effect these growth factors have on vessel growth.[29,31–33,39] Each condition was allowed to sprout capillaries for 8 days postassembly, and high-resolution phase contrast images were then taken. Growth medium was changed every 48 h in

room air for all conditions with samples taken at various time points up to 192 h and stored at −80°C for protein analysis.

Stimulating stromal cells with hypoxia

Fibrin tissue constructs were assembled according to the protocol and organized into the following experimental groups: control, 20% conditioned medium (CM), and 1% CM. CM conditions indicate that the HUVECs were incubated at 20% O_2 throughout the experiment while being supported with CM from NHLFs incubated at the indicated O_2 tension. Similar to the hypoxia induction experiments outlined above, each condition was further divided into two treatment groups: (1) maintained with exogenous VEGF and bFGF (+GF) and (2) maintained without exogenous VEGF and bFGF (−GF). Tissue constructs containing a fibroblast monolayer, treated with the growth factors VEGF and bFGF, served as a positive control. Fibrin tissues within the CM groups were constructed with EC-coated beads embedded in a 2-mm-thick fibrin gel without a fibroblast monolayer. Six tissue constructs were made for each condition, resulting in a total of 36 tissues for the six different conditions.

CM was sourced from fibroblasts incubated at various O_2 tensions. NHLFs were grown to 80% confluency in T-75 tissue culture flasks and maintained with FGM-2 (Lonza). Twenty-four hours before fibrin tissue assembly, FGM-2 was changed to EGM-2, and the flask incubated at the desired O_2 (e.g., 20% or 1%) tension at 5% CO_2 and 37°C. Medium was conditioned by the fibroblasts for 24 h before being applied to ECs. Samples of the CM were taken at 0, 12, and 24 h, and frozen at −80°C for protein analysis. Postconditioning, the medium was syringe filtered to remove any suspended fibroblasts and applied immediately to the appropriate fibroblast-free tissue construct. Growth medium was changed every 24 h for both the fibroblasts and ECs for the duration of this 8-day experiment.

Hypoxia step challenge

Following the protocol above, fibrin tissue constructs were assembled and assigned to one of two groups, control or step. Fibrin tissue constructs from the control group were maintained in a standard incubator, while those in the step group were transferred to hypoxic (1% O_2) incubator, representing a step change in the O_2 tension. Both groups of tissues contained a fibroblast monolayer, and were maintained with complete EGM-2 (i.e., formulated with all aliquots of growth factors including VEGF and bFGF). The complete fibrin gels from each group were allowed to form capillaries in culture for 8 days under normal conditions (i.e., room air, 5% CO_2, and 37°C). On day 8, images were taken of the capillary networks in each group. Images were taken again on days 12 and 16. Growth medium was changed every 48 h in room air with medium samples taken from each condition at various time points up to 192 h after the hypoxia step challenge and stored at −80°C for later analysis. Eighteen tissues were made for each condition, resulting in a total of 36 gels for this experiment.

Images and processing

Tissue constructs were imaged using phase contrast microscopy. Of the approximately 250 beads per experimental well, 10 beads were randomly selected and imaged at low power (10×) and saved as high-resolution files (*.tif). Each gel was scanned by moving the field of view from top left to the bottom right in 1 mm increments. The first 10 isolated beads (i.e., not exhibiting anastomoses with a neighboring bead) were imaged and quantified. Image processing software (Scion Image, Frederick, MD) was utilized to quantify the capillary/bead images. Capillary formation was assessed using the total capillary network length (sum of all vessel segments demonstrating visible lumen) and the total number of vessel segments per bead. These parameters were measured directly from the high-resolution images using the measurement tool of Scion Image. The personnel quantifying the images were blinded to the experimental conditions.

Enzyme-linked immunoabsorbent assay (ELISA)

Human VEGF-A and bFGF concentrations were measured in CM samples from the fibrin tissue constructs or NHLFs after stimulation from various O_2 tensions. ELISA kits for VEGF-A and bFGF were purchased from R&D Systems (Minneapolis, MN). Protein concentrations were determined in a 96-well plate per manufacturer's instructions.

Statistics

Data are presented as mean ± standard error of the mean. Data were analyzed using one-way analysis of variance (ANOVA) with Student Newman Keuls posttest analysis for multiple comparisons. Data were considered significant at $p < 0.05$.

Results

Hypoxia affects angiogenesis in vitro

O_2 tension has a significant effect on capillary formation *in vitro*. Figure 1 shows representative bright field images of capillary formation in each condition, while Figure 2A, B is the quantification. Figure 2C, D presents the VEGF and bFGF concentrations. Overall, the control condition (20% O_2 + GF) produced significantly more capillaries than the other conditions. One percent O_2 severely limited angiogenesis regardless of the presence of growth factors.

Figure 2C shows VEGF concentrations at various time points up to 192 h. VEGF is clearly produced endogenously in our coculture model of angiogenesis as evidenced by the increase in the concentration in all conditions over. In the absence of exogenous growth factors, 1% O_2 enhances VEGF concentration in our tissue model relative to control by 48 h, which continues for 192 h resulting in a threefold increase in VEGF concentration. Figure 2D shows the concentration of bFGF at the indicated time points. Unlike VEGF, bFGF concentration decreases rapidly when present initially at all O_2 tensions. When exogenous VEGF and bFGF are not added, there is no detectable bFGF in the culture medium over 192 h for both conditions.

Stimulating stromal cells with hypoxia enhances angiogenesis

To determine if capillary formation inhibition observed at low O_2 tensions is due to exposure of the ECs to chronic hypoxia, the fibrin coculture model was separated, allowing

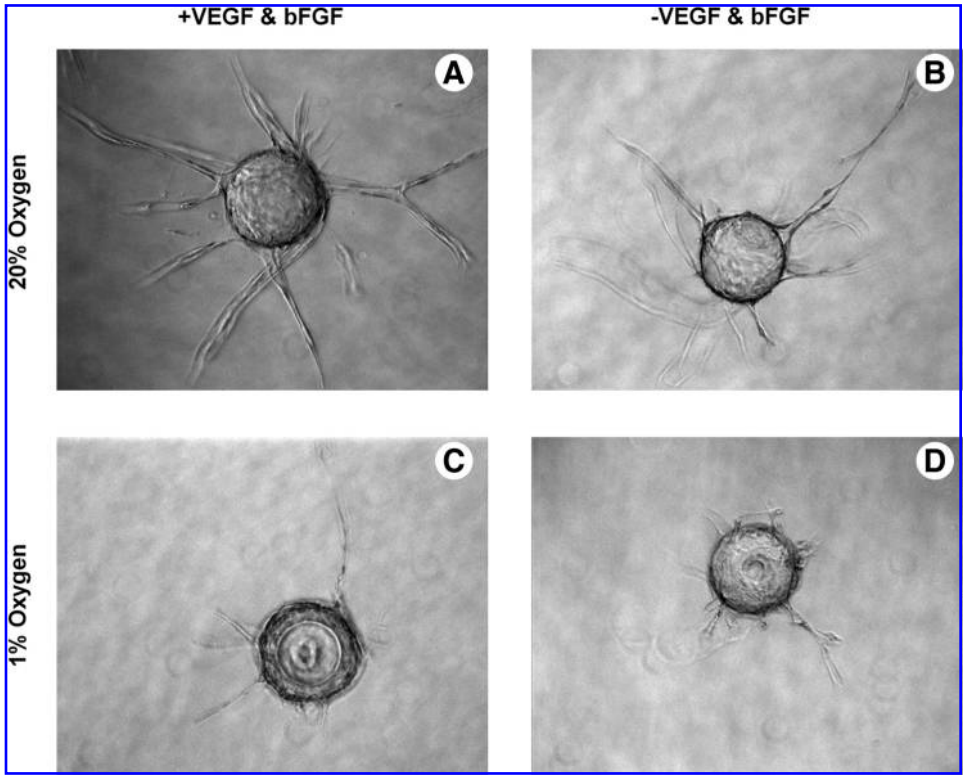

FIG. 1. *In vitro* capillary formation with decreasing O_2 tension. Representative (i.e., based on the mean of each condition) images of capillary vessel were taken after 8 days in culture and used to generate Figure 2. O_2 tension decreases from room air (20% O_2) to 1% vertically. Panels (**A**) and (**C**) show capillaries formed in the presence of the growth factors VEGF and bFGF. Capillary growth without these factors present is shown in panels (**B**) and (**D**).

each cell type to be maintained in different O_2 tensions. EC-coated beads in the fibrin slab were incubated at 20% O_2 and maintained with CM from fibroblasts at various O_2 tensions. Figure 3 is a collection of representative images of capillaries formed under each condition. The image quantification and growth factor concentrations are shown in Figure 4. In this new configuration, CM supplemented with exogenous VEGF and bFGF adequately supports capillary formation; in fact, maximal capillary growth occurred when ECs were maintained with CM from fibroblasts incubated at 20% O_2 and growth factors added exogenously (Fig. 4A). In addition, there is a near fourfold increase in vessel formation at 1% O_2 when comparing CM versus coculture (Fig. 4A compared to Fig. 2A). Nonetheless, reducing O_2 tension continues to limit capillary growth.

Figure 4B shows the number of vessel segments in the network for each condition. The trend is similar to total vessel length (Fig. 4A). However, the average vessel length (total vessel length divided by the number of vessels) decreases from 148 μm in coculture to 109 μm with CM at 20% O_2. The increase in the number of vessels combined with the decrease in average vessel length indicates that CM enhanced branching relative to the control. This can be observed qualitatively by comparing Figure 3C to Figure 3A.

VEGF and bFGF protein concentrations over a 24-h period of fibroblast conditioning are shown in Figure 4C and D. For consistency with the other experiments, time zero represents time at which the medium is transferred to the HUVEC culture to stimulate angiogenesis. VEGF concentration in the fibroblast CM increases dramatically (>3-fold) by 12 h at 1% O_2. The initial presence of VEGF and bFGF in the growth medium does not impact the VEGF concentration profile (i.e., VEGF remains relatively constant for 20% O_2, and increases significantly for 1% O_2). When added exogenously, bFGF concentration is rapidly reduced by the fibroblasts in isolation to near zero, as well as the coculture although at a slower rate (Fig. 4D). bFGF concentration is negligible when not added exogenously under all conditions.

Step change in O_2 tension causes vessel regression

To gain insight into how this model system may respond once implanted into a host's wound bed, which is known to be hypoxic, tissue constructs were allowed to prevascularize for 8 days before transfer to a hypoxic environment. Figure 5 shows representative images of capillary formation over a 16-day period. During the first 8 days at 20% O_2 the ECs sprout, migrate, and form robust capillaries consistent with Figure 1A and Figure 3A. The vessels continue to grow and develop over the duration of the experiment (days 8–16, Fig. 6A, B). The 1% O_2 step challenge causes vessel degradation by day 12 (Fig. 6A, B). VEGF concentration increases in both conditions, although the hypoxia step challenge results in a near twofold increase in VEGF concentration over the control. In contrast, bFGF concentration rapidly decreases in the first 12 h (consistent with our earlier results); however, by 96 h the 1% O_2 condition stimulates an increase in bFGF concentration (~13 pM), which is significantly increased over the control condition, but does not reach the concentration of the exogenously added bFGF (~20 pM).

Discussion

Developing functional capillary networks is a fundamental challenge facing tissue engineers. Without functional vasculature, engineered tissues must rely on net diffusion to deliver nutrients and remove cellular waste based on con-

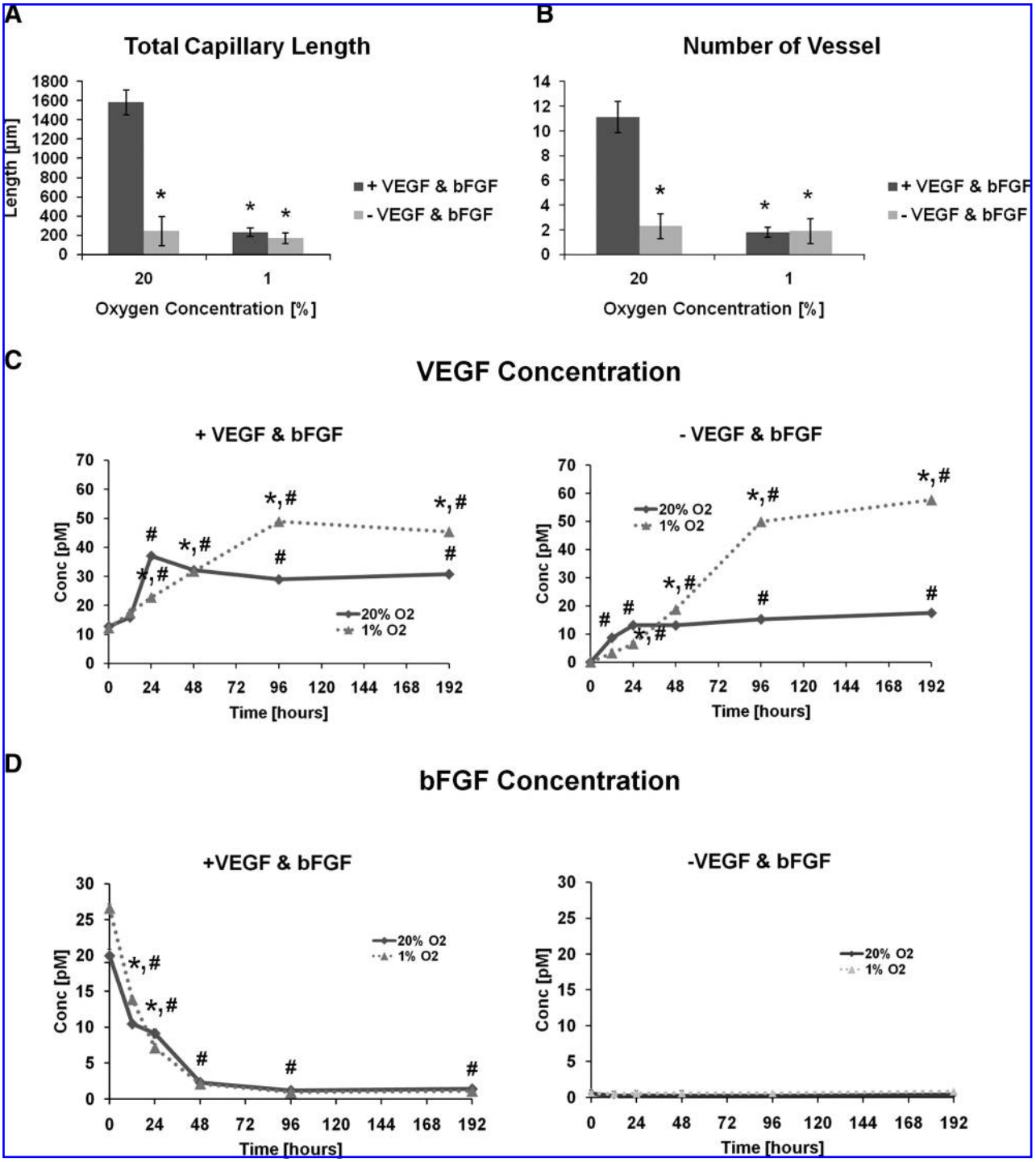

FIG. 2. Hypoxia inhibits capillary formation regardless of exogenously added VEGF and bFGF. (**A**) Total capillary length, (**B**) total number of vessels, and (**C**) VEGF and (**D**) bFGF concentration over time are shown for 20% and 1% O_2 tensions. *Significantly different ($p < 0.05$) compared to the control condition (20% O_2 + VEGF and bFGF). #Significantly different from control condition initially ($t = 0$).

centration gradients. Our previous work established a maximum diffusion distance (~1.8 mm) between a fibroblast monolayer and ECs that is critical for optimal capillary formation, and demonstrated that tissue thickness could reach 8 mm as long as the distance between the fibroblasts and ECs did not exceed 1.8 mm.[34] Further, *in vitro* we observed nearly constant levels of O_2 within the tissue,[34] indicating that hypoxia was not contributing to vessel formation. By introducing hypoxia into the tissue model, our current study is able to make the following observations: (1) angiogenesis is severely limited when both ECs and fibroblasts are exposed to hypoxia (1%) despite a significant increase in VEGF, and

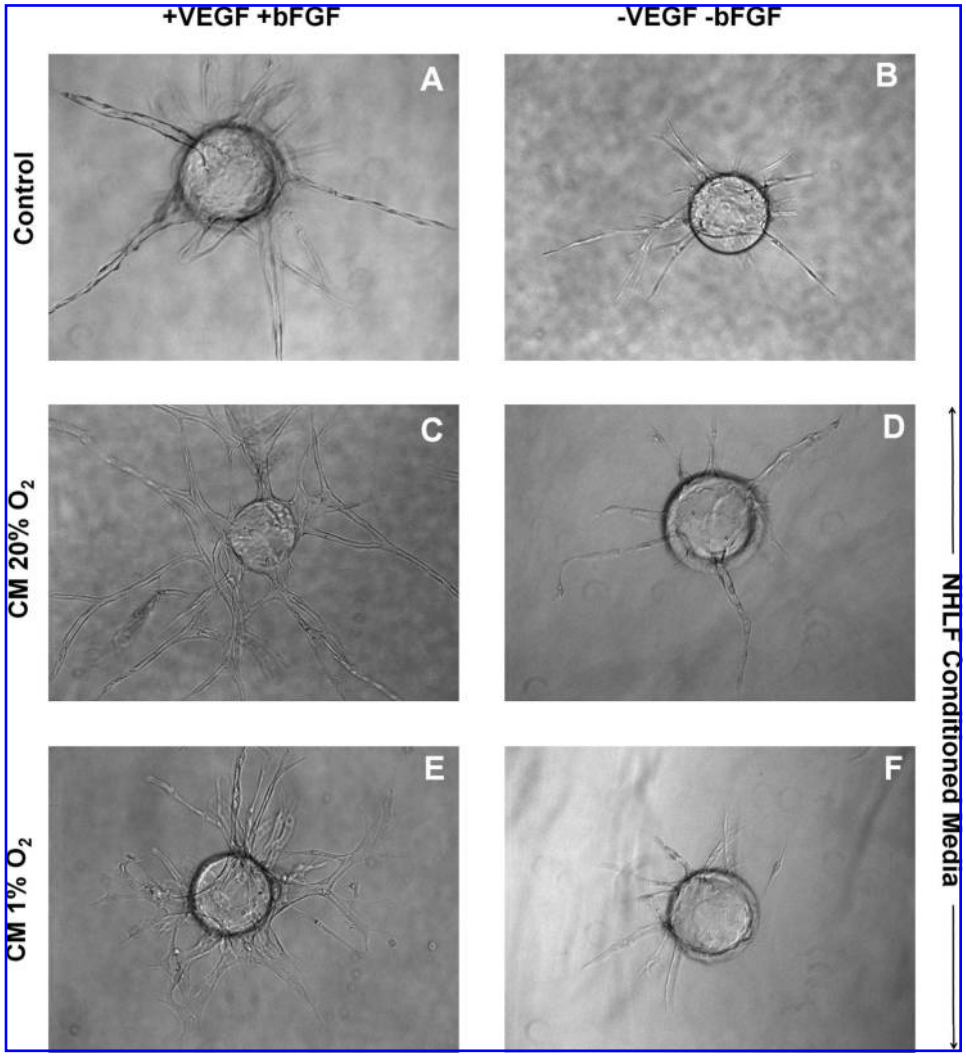

FIG. 3. Capillary vessel formation in the presence of fibroblast CM. Representative images of capillaries formed in the coculture model (**A, B**), or ECs cultured at 20% O_2 maintained with CM from fibroblasts cultured at 20% and 1% O_2 tensions (**C–F**). Capillaries grown in the presence or absence of VEGF and bFGF are shown in the left side and right side columns, respectively.

even when both VEGF and bFGF are added exogenously; (2) medium conditioned from fibroblasts supports capillary formation, but only when VEGF is added exogenously (concentration >10 pM), and maximal capillary formation occurs when the fibroblast CM is generated under 20% O_2. From these observations, we conclude that the physical presence of a stromal cell (only CM is required) is not necessary to create three-dimensional capillary networks, and that soluble fibroblast-derived mediators other than VEGF are produced under hyperoxic (20%) conditions leading to maximal capillary growth. Identifying these fibroblast-derived factors may lead to improved *in vitro* capillary formation and obviate the need for stromal cells to prevascularize an implantable soft tissue.

Hypoxia limits angiogenesis in vitro

Numerous studies implicate hypoxia as the biologic stimulus for angiogenesis.[40–51] HIF is a transcription factor that regulates the cellular response to hypoxia. Reduced O_2 tension stabilizes the HIF-1α subunit allowing heterodimerization with HIF-1β to act on hypoxic responsive elements on the target gene.[44,45,52] The result is increased growth factor production, such as VEGF, bFGF, and TGF-β1. VEGF is thought to be the primary cytokine responsible for initiating angiogenesis. It is expressed by numerous cells (in particular, stromal cells such as fibroblasts), induces vascular permeability, and acts as a potent endothelial mitogen and survival factor.[13,53–56] bFGF was isolated in angiogenic tumors and works synergistically with VEGF to promote cell survival in response to hypoxia, proliferation, chemotaxis, and VEGF/VEGFR2 upregulation in ECs.[52–54,57]

While VEGF gene expression appears to be increased in all cell types exposed to hypoxia, the expression of bFGF is more cell specific. For example, hypoxia stimulates an increase in bFGF gene expression in cortical neurons[28] and cancer cells,[58] but does not stimulate expression in vascular smooth muscle cells.[59] In addition, it has been shown that the ability of HUVECs to form cord-like structures *in vitro* is dependent on HUVEC-derived bFGF.[52] Taken together, these observations are consistent with our results. In our model system, reducing the O_2 tension generally limits capillary growth. The fibroblasts respond to hypoxia (1%) by amplifying VEGF production, but bFGF is not produced (Fig. 4C). Lung fibroblasts, a stromal cell similar to smooth muscle cells, do not upregulate production of bFGF in our culture

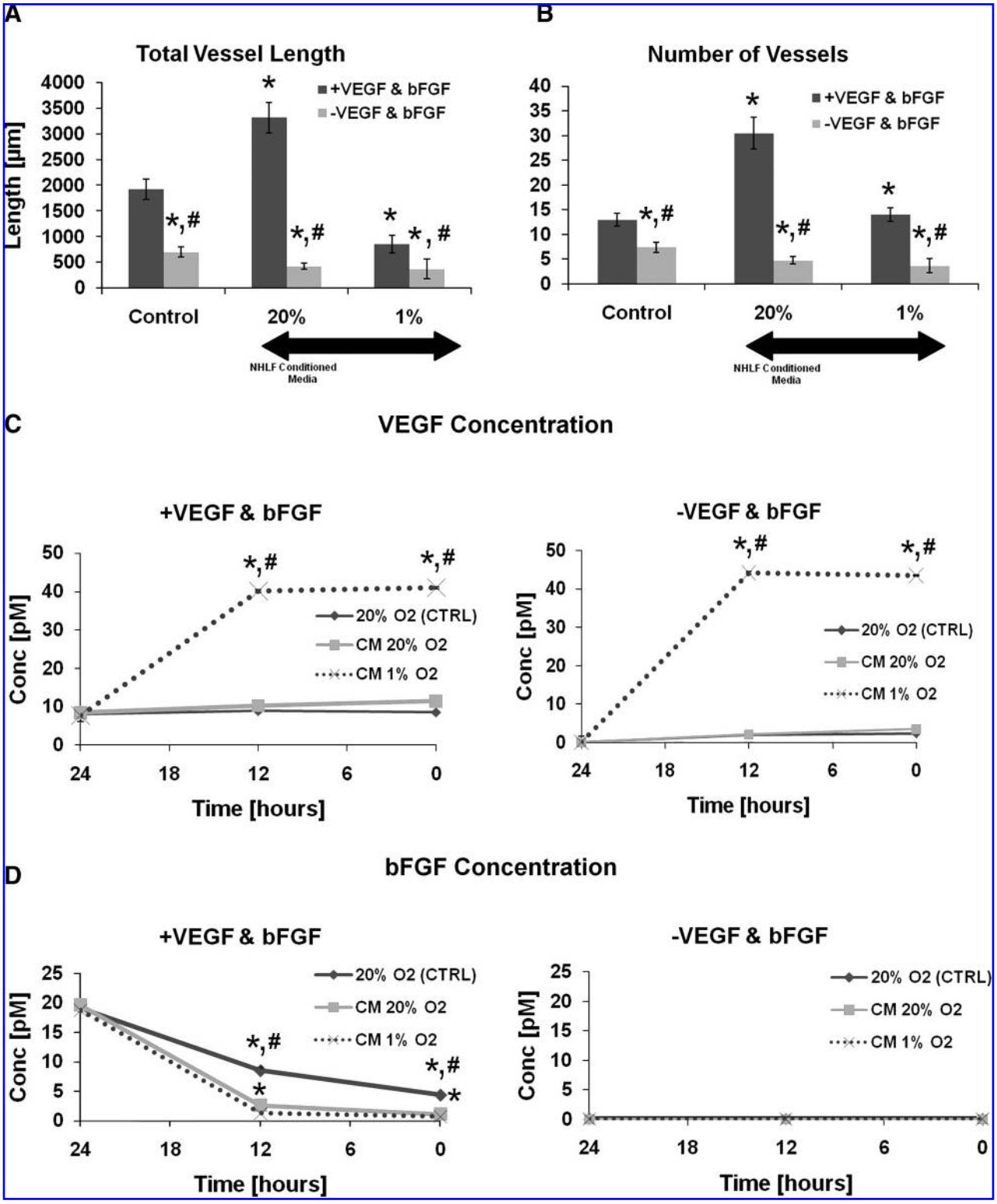

FIG. 4. Fibroblast CM enhances capillary development. (**A**) Total capillary length, (**B**) total number of vessels, and (**C**) VEGF and (**D**) bFGF concentration over time are shown for conditions corresponding to the images in Figure 3. *Significantly different ($p < 0.05$) compared to the control condition. #Significantly different from control condition initially ($t = 0$).

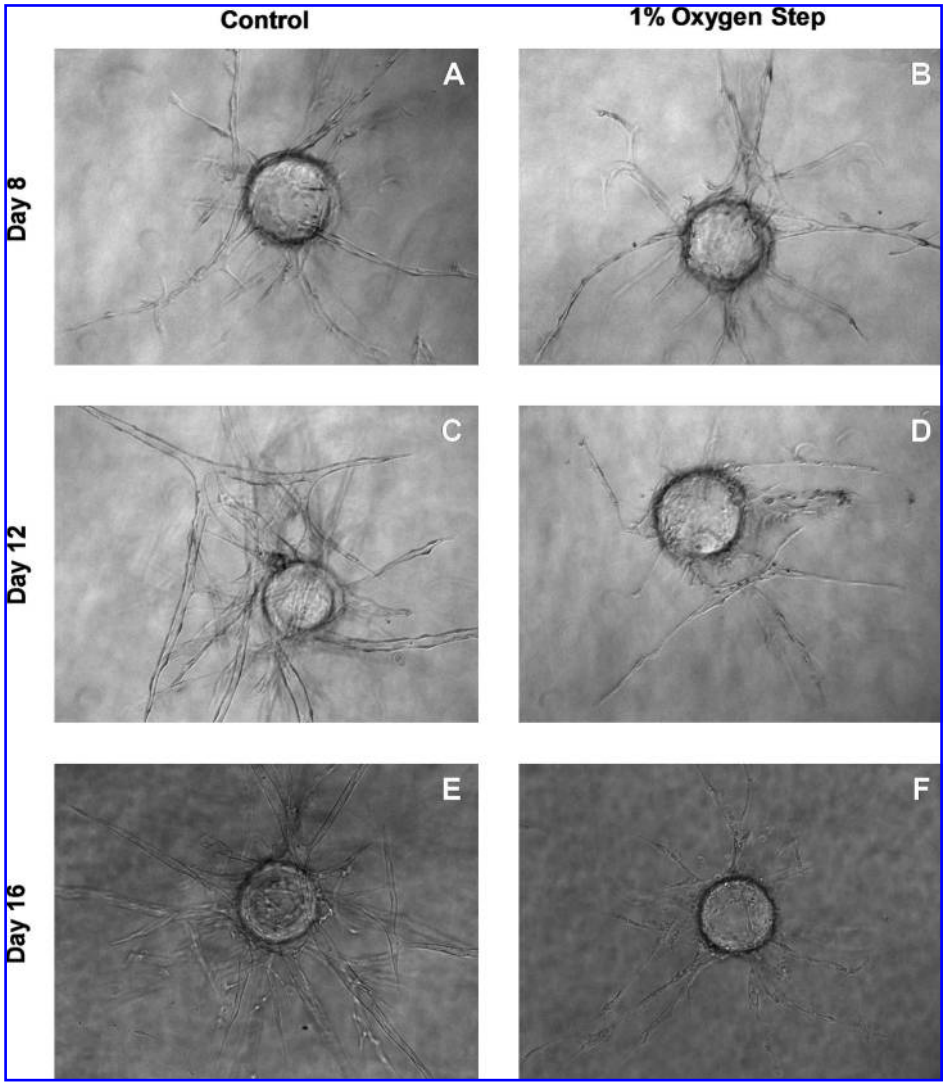

FIG. 5. Images of tissue vascularization after a 1% hypoxic step challenge. Images show representative capillary formation from tissue constructs that were maintained in culture at 20% O_2 for 16 days (**A, C, E**) or subjected to hypoxic challenge at day 8 and maintained at 1% O_2 for duration of the experiment (days 9–16; **B, D, F**). Images taken at 4-day intervals are displayed vertically.

system, and the presence of bFGF initially does not seem to be necessary for capillary growth as evidenced by a near zero concentration in the CM at the time of transfer to the HUVECs. Small (<2 pM) levels of bFGF may be necessary for capillary growth, and the HUVECs in our system are potentially a source.[52] Further, the concentration profiles of VEGF and bFGF (Figs. 2C and 4C) suggest that they are not playing a role in the reduction in capillary formation due to hypoxia. This reduction can be partially explained by ECs exposure to hypoxia. Culturing the ECs at 20% improved angiogenesis; however, the trend (total network length decreases with O_2 tension) in vessel growth remains. This finding is consistent with our previous work that identified the existence of a diffusion limited soluble fibroblast-derived mediator(s) that is critical for *in vitro* angiogenesis.[34]

Hypoxia is loosely defined as a reduction in O_2 tension that results in a shift to anaerobic metabolic processes.[60] In our system, room air (20% O_2 or 160 mmHg) as well as 10% O_2 (80 mmHg) are both considered hyperoxic relative to a healing wound bed (15–40 mmHg) or normal interstitial tensions (40 mmHg) (42). O_2 partial pressures between 5 and 15 mmHg (>1–2%) are considered hypoxic in tissues,[42,60] and several reports have used up to 3% O_2 tension to simulate hypoxic conditions.[40–42,46–48,50,61–64] Hence, relative to normal interstitial tissue, our data demonstrate substantial capillary growth when both ECs and stromal cells are under hyperoxic conditions (20% O_2). Although this may be counterintuitive, emerging research shows that hyperoxia could be used to improve wound healing by stimulating angiogenesis. Hopf *et al.* recently demonstrated that increasing O_2 tension improved angiogenesis in a Matrigel plug in a dose-dependent manner after being implanted in mice.[65] In addition, hyperoxic and hyperbaric treatment resulted in a 20% increase in perfusion of an ischemic wound compared to a nontreated wound.[66] These results indicate the existence of HIF-independent mechanisms for angiogenesis induction that may be active in our system, and thus might be exploited to enhance angiogenesis in engineered tissues.

Hypoxic stimulation of stromal cells

In vivo, injury disrupts blood flow leading to local O_2 gradients within the tissue. Cellular O_2 consumption in the damaged area leads to rapid hypoxia, while the uninjured tissue surrounding the wound remains normoxic, establishing spatial O_2 gradients. Similar gradients are created for

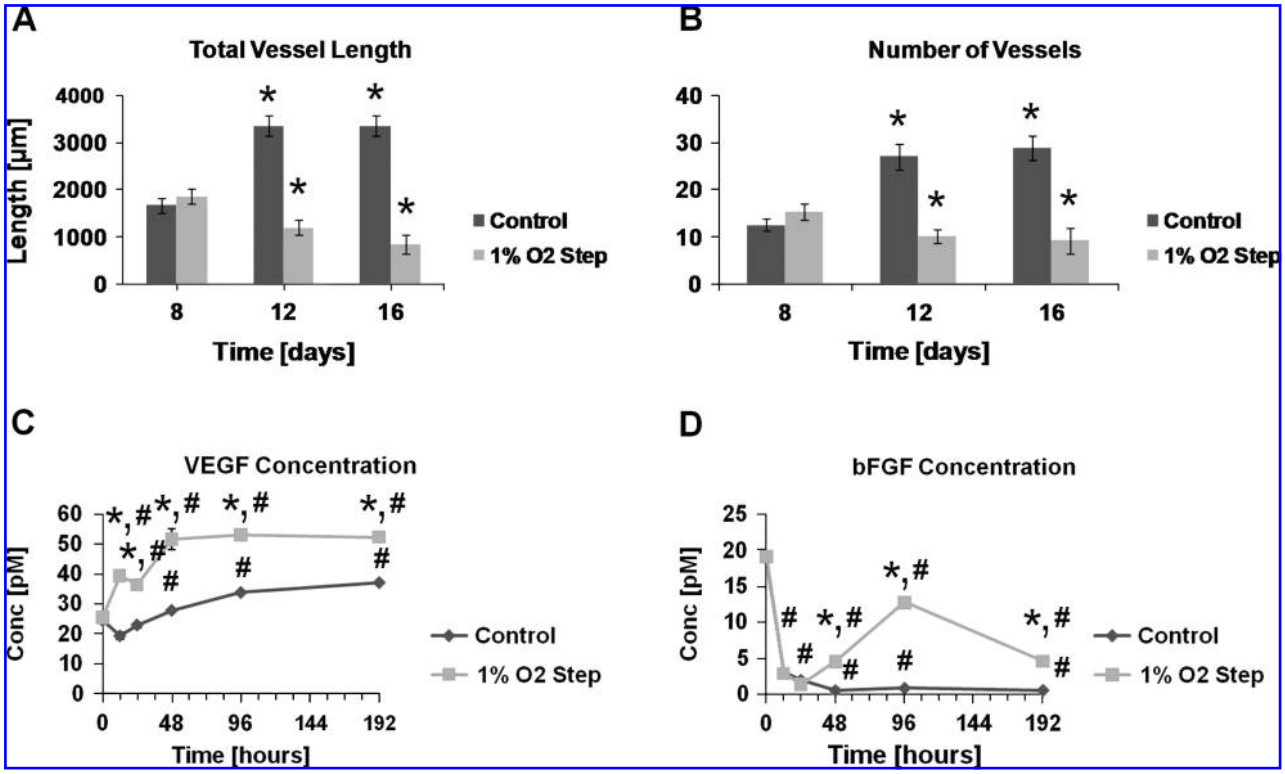

FIG. 6. Hypoxic step challenge significantly attenuates vessel formation. (**A**) Total capillary length, (**B**) total number of vessels, and (**C**) VEGF and (**D**) bFGF concentration over time are shown for conditions corresponding to the images in Figure 5. *Significantly different ($p < 0.05$) compared to the control condition. #Significantly different from control condition initially ($t = 0$).

soluble factors (i.e., VEGF and bFGF) as cells respond to hypoxia and increase production. ECs respond to these factors by initiating angiogenesis (i.e., increasing vascular permeability, proliferation, and migration).[42,52,67] Due to their proximity to intact vasculature in the undamaged normoxic region, ECs are not generally exposed to chronic hypoxia *in vivo*. In our study, when both HUVECs and fibroblasts were exposed to hypoxia, capillary growth was severely limited. However, culturing the ECs in a normoxic environment and treating them with CM from fibroblasts exposed to hypoxia enhanced capillary growth. At 1% O_2 tension, switching from coculture to CM produced a fivefold increase in vessel formation (Fig. 2B compared with Fig. 4B).

VEGF concentration increases in media conditioned with fibroblasts at 1% O_2; however, angiogenesis is still reduced compared to other conditions (i.e., maximal capillary growth occurred when CM from fibroblasts at 20% O_2 was utilized). Although the ECs migrate into the fibrin matrix, they fail to form mature capillaries. bFGF expression is not effected by hypoxia throughout these experiments. Regardless of the culture configuration, hypoxia continues to limit capillary growth, while it does not appear responsible for changes in VEGF or bFGF concentration profiles in this model. This indicates that reductions in capillary formation cannot be attributed to either VEGF or bFGF, and that other fibroblast-derived mediators in this system are hypoxia sensitive.

In both culture configurations, the highest O_2 concentration (20%) produced the largest vessel networks. This supports the existence of a fibroblast-derived soluble factor(s), other than VEGF or bFGF that significantly enhances angiogenesis under hyperoxic (20%) conditions. However, it is clear that either the production or activity of this fibroblast mediator(s) depends on the local O_2 tension, as evidenced by the significant reduction in capillary formation when fibroblast CM is generated under 1% O_2. Hence, when implementing strategies to generate vascularized tissues, the focus on VEGF and bFGF as the sole mediators of angiogenesis[11,13–15,17,68–74] may not be adequate or optimal.

Generating capillaries in the absence of fibroblasts within the *in vitro* tissue construct would potentially facilitate the creation of larger tissue dimensions. Our current results demonstrate that CM from fibroblasts is not only adequate, but leads to an increase in length and branching of the capillary network. Removing the fibroblast simplifies the system in two important ways. First, the metabolic demands of the tissue construct are reduced. Numerous reports have demonstrated that the size of an engineered tissue is inversely proportional to the cell density.[1,2,4,8,69,72,74–76] Therefore, reducing the number cells should promote larger tissues. Second, an autologous source of the stromal cell is avoided. Current research has shown the ability to isolate hematopoietic stem cells from adults and differentiate them into angiogenic ECs.[77,78] It is not yet clear whether the physical presence of the fibroblast is necessary to promote inosculation with the host upon implantation.

Of interest is the observation that switching to CM alters the branching pattern of the capillaries. CM from hypoxia-treated fibroblasts increased branching, leading to vessels

that were shorter and more numerous. The average vessel length decreased from approximately 150 μm in coculture to approximately 100 μm in CM, yet the number of branches increased nearly twofold. A vessel network in which distances between vessels are smaller may lead to enhanced nutrient delivery and waste removal once implanted.

Hypoxic step challenge

A successful prevascularized tissue needs to rapidly anastomose with the host circulation to insure the implant remains viable. The wound bed where implantable tissues may be placed is likely to be hypoxic.[11,23,65,79,80] To determine how our prevascularized tissue construct might respond *in vivo* to a sudden decrease in O_2 tension, we created well-formed capillary networks at 20% O_2 for 8 days, and then decreased the O_2 tension to 1%. The sudden decrease in O_2 led to capillary degradation within 4 days. Control tissues that were not transferred to the hypoxic environment continued to develop. Hypoxia stimulated VEGF production within 12 h, and a small amount of bFGF by 48 h. The hypoxia step challenge is the only experiment that induced bFGF. The increase in bFGF corresponds in time with the degradation of the capillary network, and may represent a survival response of the HUVECs.[52]

Conclusion

We have utilized an *in vitro* model of angiogenesis, consisting of HUVECs, NHLFs, and a fibrin matrix, to study the effects of hypoxia on capillary formation. ECs proliferate, migrate, and create a large network of well-formed capillaries after 8 days in culture. VEGF is produced endogenously within this model, and the concentration increases in response to hypoxia. In contrast, bFGF is only produced following a step change (decrease) in O_2 tension once the capillary network has already formed. Despite endogenous VEGF production, or the initial presence of both VEGF and bFGF, hypoxia severely limits capillary formation in this model. This limitation is partially due to exposing the HUVECs to chronic hypoxia. Once removed from hypoxia and maintained with CM from hypoxic fibroblasts, capillary formation improved. However, not only did HUVECs exposed to CM from fibroblasts at 20% O_2 generate the largest capillary network, but CM also enhanced branching. We conclude that soluble fibroblast-derived mediators other than VEGF and bFGF are necessary to produce a network of well-formed capillaries in a fibrin gel. This mediator(s) is produced under hyperoxic (20% O_2) conditions, but is sensitive to hypoxia. Hence, tissue engineering strategies to prevascularize fibrin-based tissues will need to consider growth factors other than VEGF and bFGF, and further work is required to identify the fibroblast-derived mediator(s).

Disclosure Statement

No competing financial interests exist.

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