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Endothelial Sprouting and Network Formation in Collagen- and Fibrin-based Modular Microbeads

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Abstract

A modular tissue engineering approach may have advantages over current therapies in providing rapid and sustained revascularization of ischemic tissue. In this study, modular protein microbeads were prepared from pure fibrin (FIB) and collagen-fibrin composites (COL-FIB) using a simple water-in-oil emulsification technique. Human endothelial cells and fibroblasts were embedded directly in the microbead matrix. The resulting microbeads were generally spheroidal with a diameter of 100–200 μm . Cell viability was high (75–80% viable) in microbeads, but was marginally lower than in bulk hydrogels of corresponding composition (85–90% viable). Cell proliferation was significantly greater in COL-FIB microbeads after two weeks in culture, compared to pure FIB microbeads. Upon embedding of microbeads in a surrounding fibrin hydrogel, endothelial cell networks formed inside the microbead matrix and extended into the surrounding matrix. The number of vessel segments, average segment length, and number of branch points was higher in FIB samples, compared to COL-FIB samples, resulting in significantly longer total vessel networks. Anastomosis of vessel networks from adjacent microbeads was also observed. These studies demonstrate that primitive vessel networks can be formed by modular protein microbeads containing embedded endothelial cells and fibroblasts. Such microbeads may find utility as prevascularized tissue modules that can be delivered minimally invasively as a therapy to restore blood flow to ischemic tissues.

Graphical abstract

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1. Introduction

It is estimated that two million Americans suffer from critical limb ischemia (CLI) caused by peripheral arterial disease, chronic kidney disease, and severe diabetes [1–3]. CLI occurs when there is a poor supply of oxygenated blood to the lower extremities of the body due to artery blockage. CLI is a chronic condition and current treatments are aimed mainly at preventing progression of the disease or salvaging existing vasculature to provide partial flow to the affected limb. However, there is a need for more permanent and effective solutions that can create new vasculature to provide sufficient oxygen and nutrients to cells in the affected tissue, and thereby prevent tissue necrosis and amputation.

A variety of strategies have been investigated to improve tissue vascularization [4]. Delivery of vascular endothelial growth factor (VEGF) is a direct approach based on the known ability of this signaling molecule to stimulate endothelial cell recruitment and subsequent neovessel formation [5–7]. Other growth factors that facilitate and increase capillary formation include basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and transforming growth factor beta (TGF-beta) [8–11]. Gene delivery has also been used to upregulate production of pro-angiogenic factors [12, 13]. A challenge in applying gene and/or growth factor delivery is determining the dosage needed to improve blood perfusion while preventing overproduction of leaky and disorganized vessels, which can occur at high dosages [5, 14, 15].

Attempts have also been made to re-vascularize ischemic tissue using transplanted cells in animal studies as well as in patients with progressed ischemic conditions [16, 17]. Although cell-based therapies have the potential to treat CLI, protocols for selection and delivery of these progenitor cells via injection into ischemic regions create challenges in terms of cell survival and engraftment. Patients are also less likely to respond to this treatment after a single dose, emphasizing the importance of optimizing the dose as well as the cell functionality being delivered to the patient [18].

Prevascularization of engineered tissues is a strategy that has been explored to allow more rapid engraftment after transplantation. *In vitro* prevascularization is typically achieved by designing and fabricating tissue constructs using appropriate extracellular matrix materials, cell types, and culture conditions to allow self-assembly of a microvascular network [19–21], or via cell seeding of a pre-defined architecture [22]. *In vivo* prevascularization relies on implanting an engineered scaffold in a region close to an artery to allow vessel ingrowth and

network formation within the implant. A major advantage of pre-vascularized tissue constructs is that the host vasculature can rapidly inosculate with the pre-formed vascular network of the construct [23–25], thereby accelerating the wound healing and remodeling needed to restore tissue functionality [21, 26, 27]. However, a major limitation with current prevascularization strategies is the requirement for invasive surgery to implant the engineered tissue at the target site.

Fibrin is the primary structural component of blood clots and plays an important role in the provisional matrix that is remodeled during wound healing [28]. Type I collagen is a well characterized structural protein and an important component of the extracellular matrix of many tissues [29]. Each of these materials has been used separately as 3D matrices for vascularization because of their demonstrated ability to support the formation of endothelial networks [30–34]. These natural matrices provide cell-adhesion sites and can be enzymatically remodeled by cells. However, combinations have also been used to support vascularization, in part to harness their composite mechanical and biochemical functionalities [31, 35, 36].

Elongation and vascular network formation by endothelial cells embedded in fibrin, collagen, and COL-FIB composite materials has been shown to be influenced by the properties of the matrix [31, 36, 37], and by the presence of stromal cells. Mesenchymal stem cells (MSCs), smooth muscle cells, and fibroblasts are stromal cells that interact with endothelial cells and contribute to the formation, remodeling, and stabilization of blood vessels in part by providing paracrine cues [38–43].

In the present study, we developed and characterized modular protein microbeads designed as a minimally invasive, cell-based therapy for revascularization of ischemic tissue. Human endothelial cells and fibroblasts were embedded in pure fibrin and collagen-fibrin microbeads using a simple water-in-oil emulsification process. We examined how cell concentration and matrix composition influenced cell incorporation into microbeads, and characterized cell viability and proliferation in the matrix. Selected microbeads were subsequently embedded into surrounding fibrin hydrogels to study vascular network formation. The morphology and extent of endothelial sprout formation inside and outside microbeads was quantitatively assessed over time in culture. In addition, anastomosis of vessel segments from neighboring microbeads was characterized. The overall goal of this work was to combine cell-based approaches to vasculogenesis with prevascularization strategies to produce a potential therapy for the rapid and efficient restoration of blood flow to regions of tissue ischemia.

2. Materials and Methods

2.1 Cell culture

Umbilical cords were obtained from the University of Michigan Mott Children's Hospital via an IRB-exempt protocol and human umbilical vein endothelial cells (HUVEC) were isolated via methods similar to those previously described [30]. In brief, the umbilical cord was washed in phosphate buffer saline (PBS) and digested in collagenase type I solution (195 U/ml, Worthington Biochemical, Lakewood, NJ) at 37°C for 20 min. The digested

tissue was washed in PBS and subsequently centrifuged (200×g for 5 min). HUVEC were plated in tissue culture flasks and supplied with endothelial growth media (EGM-2, Lonza). After 24 hours, HUVEC were rinsed with PBS thrice to remove non-adherent cells and supplied with fresh media that was changed every 48 hours. Cells from passages 3 and 4 were utilized for experiments. Normal human lung fibroblasts (NHLF, Lonza Inc., Walkersville, MD) were cultured in Media 199 (M199, Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Life Tech). Culture media was replaced every 48 hours and cells from passages 7–12 were used in experiments.

2.2 Microbead fabrication

The process used to create protein microbeads is shown schematically in Figure 1. Two matrix formulations were used: 100% fibrin (FIB) and 40/60 wt% collagen/fibrin (COL-FIB). Collagen stock solution (4.0 mg/ml) was made under sterile conditions by dissolving lyophilized type I bovine collagen (MP Biomedicals, Solon, OH) in 0.02 N acetic acid. Fibrinogen stock solution (4.0 mg/ml clottable protein) was made by dissolving bovine fibrinogen (Sigma Aldrich, St. Louis MO) in serum-free endothelial growth medium (SFEGM-2) at 37°C. The solution was then filter sterilized after completely dissolving the protein. Equal numbers of HUVEC and NHLF were suspended in FIB or COL-FIB composite hydrogel solutions at specific cell concentrations (Low: 5×10^5 cells/ml, High: 2×10^6 cells/ml). To make 1.0 mL of cellular FIB hydrogel solution, the following components were added to the cell pellet and mixed thoroughly: 255 μ L SFEGM-2, 100 μ L of FBS (10% final), 20 μ L of 50 U/mL thrombin (1 U/mL final), and 625 μ L fibrinogen stock solution (2.5 mg/mL final). To make 1.0 mL of cellular COL-FIB composite hydrogel solution, the following components were added to the cell pellet and mixed thoroughly: 93 μ L SFEGM-2, 100 μ L FBS (10% final), 100 μ L of 5X Dulbecco's Modified Eagle Medium (DMEM (10% Final), 12 μ L of 87.5 mM glyoxal (1 mM Final, Sigma) for crosslinking collagen, 50 μ L of 0.1 N NaOH to neutralize acidic collagen, 20 μ L of 50 U/mL thrombin (1 U/mL final), 250 μ L collagen stock solution (1.0 mg/mL final), and 375 μ L fibrinogen stock solution (1.5 mg/ml final).

To create protein microbeads, 3.0 mL of matrix/cell suspension was quickly added to a stirred bath (600 rpm) containing 75 mL of 100 cSt polydimethylsiloxane (PDMS, Dow Corning) cooled in an ice bath. Emulsification proceeded for 5 min, and the temperature of the PDMS bath was then increased to 37 °C to induce gelling for an additional 25 min. The mixture of PDMS and formed microbeads was then transferred to 50 mL centrifuge tubes and 5 mL of PBS containing 0.1% L101 surfactant (PBS-L101; BASF, Florham Park, NJ) was added to each tube and mixed gently by inverting for 5 min. The mixture was then centrifuged at 200xg for 5 min. The PDMS layer was carefully removed and then pelleted microbeads were washed again using PBS-L101. Once the microbeads were thoroughly washed, they were supplied with an appropriate amount of culture medium (EGM-2) and used for further experiments.

2.3 Characterization of cell incorporation and microbead morphology

The number of cells incorporated within microbeads was determined by staining with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies). The FIB and COL-FIB samples were collected and fixed with zinc-buffered formalin solution (Z-fix, Anatech, Battle Creek, MI) for 5 min. Fixed samples were washed in PBS and stained with 10 nM DAPI solution for 10 min. Bright field and fluorescent images were taken using an optical microscope (Olympus IX81, Olympus, Center Valley, PA). The number of cells per microbead and microbead diameters were determined from these images using ImageJ (National Institutes of Health, Bethesda, MD). At least 100 randomly selected microbeads per condition and per experiment were used for image analysis to determine microbead diameter, cell distribution, and number of cells incorporated into the microbeads.

2.4 Cell viability and proliferation assays

The viability of cells encapsulated in microbeads and in bulk gels was determined using the Live/Dead® staining kit (Life Technologies). Bulk gel samples with the same protein and cell concentration as the microbeads were also prepared by allowing the hydrogel/cell mixture to gel in 24-well plates (500 µL per well) at room temperature for 5 minutes, followed by an additional 20 to 25 min at 37 °C in an incubator. Samples were collected, washed in 10 mM PBS and re-suspended in PBS containing 1 µM calcein-AM and 2 µM ethidium homodimer for 45 min at 37°C. Samples were then imaged using a fluorescence microscope.

Both cell seeded COL-FIB and FIB microbeads were embedded in fibrin gels after their fabrication process. To embed microbeads in gels, one tenth of a 3 mL microbead preparation was collected and pelleted to make three fibrin hydrogels with microbeads per time point (~500 µL per hydrogel). These hydrogels were made by adding the following components to the microbead pellet and mixing thoroughly: 382.5 µL SFEGM-2, 150 µL of FBS (10% final), 30 µL of 50 U/mL thrombin (1 U/mL final), and 937.5 µL fibrinogen stock solution (2.5 mg/mL final). Hydrogels were molded into standard 24-well culture plates, and were left at room temperature for 5 min before being placed in an incubator for 25 minutes at 37°C. One milliliter of EGM-2 was added to each hydrogel containing the cellular microbeads. Culture medium was replaced the next day and then on every second day for the duration of the culture period.

Cell proliferation was determined by quantifying total DNA in samples collected at specific time intervals. To isolate the total DNA from the samples, fibrin gels containing microbeads were digested in 10 mM Tris-HCL/0.4 M Guanidine hydrochloride solution for 3 hours. The digested samples were then centrifuged at $10,000 \times g$ for 10 min and the supernatant containing the DNA was aspirated, and then diluted with water to reduce Guanidine-HCl interference with the DNA assay. DNA content was determined using the PicoGreen® DNA assay kit (Invitrogen). Calf thymus DNA standards were used to generate standard curves.

2.5 Imaging and analysis of endothelial sprouts

To distinguish the microbeads from the 3D fibrin environment in which they were embedded, FIB and COL-FIB microbeads fabricated as described above were subsequently

coated with a FITC-fibrinogen layer by incubating them in FITC-fibrinogen (9 µg/mL, Life Technologies) in EGM-2 at 37 °C for 25 min prior to embedding within the larger hydrogel. The same embedding process utilized for the proliferation assay (Section 2.4) was employed to incorporate the cellular microbeads within acellular fibrin hydrogels. To visualize vascular sprouts, the endothelial cell-specific marker Ulex Europaeus Agglutinin I (UEA-I, Vector Laboratories, Burlingame, CA) was used. In brief, samples were collected at specified time intervals, washed twice in PBS, and fixed in zinc-buffered formalin (Z-fix, Anatech) at room temperature. Fixed samples were then washed and incubated in staining solution containing 1% BSA, 20 µg/ml rhodamine-labeled UEA-I, and 10 nM DAPI in PBS for 45 min at room temperature. Before imaging, samples were rinsed twice with PBS for 5 min to remove unbound residual stains.

Endothelial sprouts were characterized by measuring average sprout lengths, numbers of segments (branching), branch points, and lengths of each segment both inside and outside the microbeads through image analysis using a protocol similar to previous studies [30, 37, 44]. Inosculation of adjacent sprouts protruding from adjacent microbeads was assessed qualitatively. Fluorescence images were acquired using an optical microscope and randomly selected microbeads were used for quantification using ImageJ. All image analysis was performed using a blinded image evaluation method in which random images were provided to the evaluator with no information about the experimental conditions.

2.6 Statistical Analysis

Statistical analyses were performed using STATISTICA (StatSoft, Inc, Tulsa, OK) software. At least 3 independent experiments were performed for each assay and data are reported as mean ± standard error of the mean (SEM). One-way ANOVA followed by unequal N post-test was used to compare multiple conditions. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1 Cell encapsulation and retention in microbeads

HUVEC and NHLF were encapsulated at a 1:1 ratio in FIB and COL-FIB microbeads, at either low cell concentration (LC: 5.0×10^5 cells/ml) or high concentration (HC: 2.0×10^6 cells/ml). At the same starting concentration, larger microbeads contained greater total numbers of cells, as expected (Fig. 2A). The average microbead diameter was approximately 140 µm for both FIB formulations and the low cell concentration COL-FIB formulation; however the high cell concentration COL-FIB microbeads were significantly larger (approximately 200 µm in diameter, Fig. 2B, $p < 0.05$). The distribution in the number of cells per microbead of the treatment populations was broader for the high cell concentration conditions, in contrast to microbeads containing the low cell concentration, which exhibited a sharper peak and a more homogeneous distribution (Fig. 2C). Overall cell incorporation increased as higher cell concentrations were used with the same material formulation (Fig. 2D, $p < 0.05$). Microbeads with high cell concentration contained an average of 30–35 cells per microbead, while those with low cell concentration contained 5–10 cells per microbead. There was no statistical difference in the total number of cells incorporated between COL-

FIB and pure FIB microbeads, when comparing at either the low concentration or high cell concentration conditions.

3.2 Viability and proliferation of cells encapsulated in microbeads

The viability of cells encapsulated in FIB and COL-FIB microbeads in the high cell concentration (2.0×10^6 cells/ml) was quantified and compared to values from bulk macroscopic hydrogels (Fig. 3). Representative live (green)/dead (red) images of COL-FIB microbeads (Fig. 3A), FIB microbeads (Fig. 3B), COL-FIB bulk hydrogels (Fig. 3C), and FIB bulk hydrogels (Fig. 3D) show uniform cell distributions in both the microbeads and the hydrogels, with no spatial variations in viability evident. Cell viabilities in both COL-FIB and FIB microbeads were high (75% and 79% viable cells, respectively), with no statistically significant differences between the formulations. However, cell viability was significantly lower in the microbeads compared to their bulk hydrogel counterparts, which exhibited 87% and 85% viable cells, respectively (Fig. 3E).

COL-FIB and FIB microbeads made at the high cell concentration (2.0×10^6 cells/ml) were embedded in surrounding 2.5 mg/ml fibrin hydrogels to examine cell proliferation and endothelial sprout formation. Figure 4A shows a schematic of the protocol used for cell proliferation studies. Microbeads were visualized inside the larger fibrin matrix using brightfield and fluorescent microscopy (Fig. 4B). In these images, FIB microbeads were embedded in a FIB hydrogel immediately after fabrication and were cultured for 7 days before imaging. In the fluorescence image, microbeads were coated with a FITC-fibrinogen layer to distinguish the microbead matrix from the surrounding fibrin. At day 0, both microbead formulations showed equal DNA content, which then increased over time in culture as cells proliferated. At day 7, there was a significant difference ($p < 0.05$) in the DNA content between FIB and COL-FIB microbeads. By day 14, the DNA content was 2.5-fold higher ($p < 0.05$) in COL-FIB microbeads than in corresponding FIB microbeads, indicating a significantly higher number of cells within these beads (Fig. 4C).

3.3 Endothelial sprouting from microbeads embedded in fibrin matrix

COL-FIB microbeads made with the lower cell concentration did not support endothelial sprouting when embedded in fibrin hydrogels (data not shown), while those made with the higher cell concentration retained the capacity for sprout formation when embedded in fibrin hydrogels. Therefore, the vasculogenic capacity of COL-FIB and FIB microbeads were compared only at the higher initial cell concentration. COL-FIB and FIB microbeads containing HUVEC and NHLF were embedded in fibrin hydrogels and cultured for 7 and 14 days (representative images shown in Fig. 5). The microbead matrix was coated with a FITC-fibrinogen layer (green) and the endothelial cells were identified by UEA-1 staining (red). In both microbead formulations, endothelial sprouts initially formed inside the microbead matrix and then invaded into the surrounding fibrin hydrogel.

The extent of sprouting both within the microbeads and in the surrounding matrix were characterized in terms of the number of segments (Fig. 6A), the average segment length (Fig. 6B), the number of branch points (Fig. 6C), and the total sprout length (Fig. 6D) (see also Tables 1 and 2, Supplementary data). Microbeads contained an average of 4 to 5 vessel

segments by the end of 7 days in culture, with no further change in the number of segments within the microbeads by day 14 (Fig. 6A, “inside microbead”). The number of segments that extended from the FIB microbeads was significantly higher compared to COL-FIB microbeads at both time points. A higher number of segments protruding the COL-FIB microbeads was found with an additional week of culture (Fig. 6A, “outside microbead”). By day 14, the total number of vessel segments per microbead was 10–14, and this measure was significantly higher in FIB microbeads than in COL-FIB microbeads at both day 7 and day 14 of culture (Fig. 6A, “total per microbead”).

The average vessel segment length inside microbeads was approximately 90 μm , with no significant differences across matrix formulations or time points (Fig. 6B, “inside microbead”). Outside the microbeads, longer segments were produced by FIB microbeads than COL-FIB microbeads ($p < 0.05$), and segment length increased significantly outside FIB microbeads between 7 and 14 days (Fig. 6B, $p < 0.05$). Both COL-FIB and FIB microbeads on average contained at least one branch point, and the number of branch points was significantly higher in FIB microbeads at day 14 (Fig. 6C, $p < 0.05$), compared to the other formulation and time point. The number of branch points outside the microbeads and the total number of branch points were also significantly higher in FIB microbeads compared to COL-FIB at both time points (Fig. 6C, $p < 0.05$).

The combination of vessel network characteristics described also resulted in differences in the total sprout network length (Fig. 6D). The average total sprout length inside microbeads was 315–460 μm , and there were no statistically significant differences between matrix formulations or time points. However, the vessel networks that penetrated into the surrounding fibrin hydrogel were significantly longer when emerging from FIB microbeads compared to COL-FIB microbeads at both day 7 and 14 (Fig. 6D, “outside microbeads”, $p < 0.05$). Total sprout length increased significantly in COL-FIB microbeads over time, but was lower than in FIB microbeads at all time points (Fig. 6D, “total per microbead”, $p < 0.05$). By day 14, the microvascular network produced by HUVEC and NHLF encapsulated within FIB microbeads approached 2500 μm in total length.

3.4 – Inosculation between endothelial sprouts from adjacent microbeads

COL-FIB and FIB microbeads embedded in fibrin hydrogels were cultured for 1 or 2 weeks. The microbead matrix was labeled green prior to the embedding process to distinguish them from the surrounding fibrin matrix, while the HUVEC were stained in red (Fig. 7). Microbeads were generally separated from each other in the surrounding gel, though some clumping was observed. As described quantitatively in Figure 6, endothelial cell networks extended from the embedded microbeads by day 7 (Fig. 7A–D), and were more prominent in the pure FIB samples. The arrows in Figure 7 show anastomoses between endothelial sprouts from adjacent microbeads. This phenomenon is evident in both microbead formulations at both day 7 and day 14 and at 4 $\times$ and 10 magnification. The nuclei of both EC and NHLF were stained in blue (Fig. 7B, D, F, and H). It can be seen that NHLF migrated out of the microbeads and into the surrounding matrix, and that endothelial sprouts extended and inosculated between microbeads. By day 14, relatively long distances (1000–2000 μm) were traversed by joined endothelial sprouts (Fig. 7E–H).

4. Discussion

Cells in most tissues in the body receive oxygen and nutrients via blood from capillaries that are spaced approximately 100–200 μm apart [24, 45, 46]. In larger engineered tissue constructs, oxygen and nutrients are consumed by cells that reside within this diffusion limit, while those cells that reside beyond the limit typically undergo apoptosis. Therefore, modular approaches are being explored to reduce diffusion limitations that occur in large engineered tissues [47–52]. One strategy has been to create very small “microtissues” with diffusion path lengths on the scale of 10–100 μm . These building blocks are designed to avoid mass transfer limitations, and in some cases can be delivered minimally invasively and assembled into larger tissue structures [53–60].

In the present study, a simple water-in-oil emulsification technique was used to fabricate protein microbeads with diameters in the range of 100–200 μm . Endothelial cells and fibroblasts were fully embedded within the microbeads. These cell types have been used widely to initiate vasculogenesis and angiogenesis [37, 61], through a process in which fibroblast-derived factors provide signals to promote and stabilize network formation by the endothelial cells [42, 43]. Collagen and fibrin were used in microbeads as extracellular matrix proteins because of their demonstrated ability to support vascular network formation [35, 36, 62]. It has been shown that the composition of the matrix affects the process of neovessel formation [31, 36], and that fibrin is a particularly permissive environment for angiogenesis [20, 30, 32]. However, most studies have been performed in bulk hydrogel materials, and the properties and dynamics of the extracellular matrix in microtissues may be different from larger-scale models. Therefore this study focused on determining a suitable cell and matrix composition to achieve sprout formation by endothelial cells within collagen-fibrin and pure fibrin microbeads.

The microbeads produced by the emulsification and collection process were generally spheroidal with a diameter of 100–200 μm , and therefore the maximum diffusion path length to a cell in the interior was approximately 100 μm . Cells were incorporated into the microbead matrix in general proportion to the starting cell concentration; however, cell loss did occur during processing. Microbeads fabricated with an initial concentration of 5.0×10^5 cells/mL contained an average of only 5–10 cells, which was not supportive of the creation of vascular networks within the microbead volume. The low cell number meant that there were insufficient endothelial cells to connect and form networks, as well as reduced paracrine signaling from the relatively few supporting fibroblasts. Therefore subsequent experiments on vessel network formation were performed using only microbeads fabricated at higher cell concentration. With a starting concentration of 2.0×10^6 cells/mL of protein matrix, microbeads contained an average of 30–35 cells. Approximately half of these cells could be expected to be endothelial cells, which provided a number sufficient to initiate the formation of small vascular structures within the microbeads.

Cell viability in microbeads was lower than in bulk hydrogels of corresponding composition, but was still in the range of 75–80%. The decrease in cell viability was most likely due to the processing required to fabricate and collect the microbeads, which included multiple wash and centrifugation steps to separate the microbeads from the silicone oil. However,

cells survived within microbeads over time in culture, as evidenced by their maintained ability to proliferate and change morphology in the matrix. Cells in both microbead types proliferated in culture, but over the 14-day period studied there was a significantly greater degree of cell proliferation in COL-FIB microbeads, as compared to pure FIB microbeads. Tracking of total DNA content did not allow discrimination of which cell types were proliferating. However endothelial network length was greater in FIB microbeads than in COL-FIB microbeads, suggesting that the increased DNA content in the latter formulation was predominantly the result of fibroblast proliferation.

Endothelial sprout formation and anastomosis between sprouts was examined by embedding microbeads in an acellular surrounding hydrogel consisting of pure fibrin. The surrounding matrix isolated the microbeads from each other and allowed the examination of endothelial network extension outside of the microbeads. Using this system, both COL-FIB and FIB materials supported endothelial network formation inside the microbead matrix, as well as their extension into the surrounding fibrin hydrogel. Over time, anastomosis of endothelial sprouts from adjacent microbeads was also observed in both microbead formulations. The extent of the internal networks was generally the same regardless of microbead composition, but the extension of sprouts into the surrounding matrix was significantly enhanced using pure FIB microbeads. The networks in FIB microbeads also were more branched with longer inter-branch segments than their COL-FIB counterparts. The longer and more extensive networks produced by FIB microbeads may have a greater chance of creating inter-network connectivity.

Endothelial cell sprouting and migration have been shown to be dependent on matrix composition, stromal cell type, and serum concentration [43, 63, 64]. Since our studies used the same stromal cell type and serum concentration in both microbead formulations, the differences in sprouting in our study are mainly attributable to matrix composition. Previous studies have shown that collagen, fibrin, and their composites support endothelial network formation to different extents [62], and these effects have been correlated to matrix mechanical properties [31]. In addition, fibrin(ogen) (through its heparin-binding domain) binds to many growth factors that stimulate angiogenesis [65] and enhance wound healing [66, 67]. In contrast, the growth factor binding affinity of type I collagen is very low since no growth factor binding sites have been identified in this structural matrix protein [68]. Therefore both compositional differences and the degree of local growth factor sequestration may explain the increased propensity of vessel networks to extend from pure fibrin microbeads.

5. Conclusion

These studies demonstrate that protein-based microbeads containing endothelial cells and fibroblasts can support the formation of vascular networks by sprouting from individual microbeads, and that adjacent networks can anastomose to produce larger structures. Pure fibrin microbeads triggered the formation of more branched and longer networks, compared to collagen-fibrin composite microbeads. The use of pure protein microbeads has the advantage that the matrix composition is simpler and more readily developed as a cell therapy. Such microbeads may find utility as a method to deliver

vascularizing modules to treat ischemic conditions, and may also be useful to study the processes of vasculogenesis and angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Microbead fabrication process

HUVEC and NHLF were incorporated in a 1:1 ratio at an initial cell density of 5×10^5 cells/ml or 2×10^6 cells/ml in either pure fibrin or collagen-fibrin (40–60) microbeads via an oil-and-water emulsification process. Resultant microbeads were subsequently embedded in fibrin hydrogels, cultured for 7 or 14 days, and then analyzed.

Fig. 2. Microbead characterization

(A) The number of cells per microbead was quantified as a function of microbead diameter. Average microbead diameters (B), cell number distributions (C), and average number of cells (D) within COL-FIB and FIB microbeads at low (LC) and high (HC) cell densities were quantified. Statistical differences ($p < 0.05$) between conditions are denoted by *, while differences between one group and the remaining groups are denoted by ***. ($n = 3$, at least 100 microbeads per n).

Fig. 3. Cell viability in microbeads

Representative live (green)/dead (red) images of cells (HUVEC:NHLF co-cultures) incorporated in (A) COL:FIB microbeads, (B) FIB microbeads, (C) COL-FIB, and (D) FIB hydrogels after their respective fabrication process. (E) Images were quantified to compare the viabilities of cells in microbeads and hydrogels. Statistical differences between conditions are denoted by * ($p < 0.05$). (n=3). Scale bar = 200 μm.

Fig. 4. Cell proliferation in microbeads

(A) Schematic depicting experimental procedure for cell proliferation studies in which either COL-FIB or FIB microbeads were embedded in FIB hydrogels and cultured for 7 and 14 days. (B) Representative bright-field and fluorescence images of FIB microbeads (green in the fluorescent image) embedded at day 0 and cultured for 7 days. (Microbeads were fabricated with FITC-fibrinogen to illustrate the location of the microbeads within the fibrin matrix in this image. However, non-fluorescent fibrinogen was employed in the actual proliferation studies.) (C) DNA content of cells embedded within COL-FIB or FIB microbeads was quantified after 1 and 2 weeks in culture. Statistical differences between microbead types at both day 7 and day 14 are denoted by * ($p < 0.05$, $n = 3$). Scale bar = 500 μm .

Fig. 5. Endothelial sprouting within and from microbeads

Representative images of HUVECs (red) and microbeads (green) in either COL-FIB (A, C) or FIB microbeads (B, D) embedded immediately after processing, and cultured for an additional 7 and 14 days within FIB constructs. Sprouts originate from within the beads and invade the surrounding matrix. Scale bar = 500 µm.

Fig. 6. Quantification of endothelial sprout lengths and branching

(A) The numbers of vessel segments, (B) average segment lengths, (C) numbers of branch points, and (D) sprout lengths for the nascent microvessel-like networks originating from COL-FIB and FIB microbeads embedded in fibrin gels at day 0, and cultured for an additional 7 and 14 days. Statistical differences ($p < 0.05$) are denoted by the following symbols: * for the differences between microbeads at D7, \$ for the differences between microbeads at D14, ^ for differences across culture time (D7 vs. D14) for microbeads of the same composition, and # for differences in culture time and microbead composition ($n = 3$).

Fig. 7. Formation of anastomoses between sprouts to form larger microvascular networks
Connections (white arrows) formed via inosculation of endothelial sprouts from adjacent COL-FIB (A, B, E, F) or FIB (C, D, G, H) microbeads embedded in FIB hydrogels immediately post fabrication, and cultured for 7 days (A–D) and 14 days (E–H). Images are 4× and 10× magnification. HUVEC are displayed in red, microbeads in green, and cell (both HUVEC and NHLF) nuclei in blue. Scale bar = 500 μm.