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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.

Statement of significance

Vascularization is critically important for tissue engineering and regenerative medicine, and materials that support and/or promote neovascularization are of value both for translational applications and for mechanistic studies and discovery-based research. Therefore, we fabricated small modular microbeads formulated from pure fibrin (FIB) and collagen–fibrin (COL–FIB) containing endothelial cells and supportive fibroblasts. We explored how cells encapsulated within these materials form microvessel-like networks both within and outside of the microbeads when embedded in larger 3D matrices. FIB microbeads were found to initiate more extensive sprouting into the surrounding ECM *in vitro*. These results represent an important step towards our goal of developing injectable biomaterial modules containing preformed vascular units that can rapidly restore vascularization to an ischemic tissue *in vivo*.

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

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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 preformed 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 (200xg for 5 min). HUVEC were plated in tissue culture flasks and supplied with endothelial growth media (EGM-2, Lonza). After 24 h, HUVEC were rinsed with PBS thrice to remove non-adherent cells and supplied with fresh media that was changed every 48 h. 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 h, 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 Fig. 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 μL of SFEGM-2, 100 μL of FBS (10% final), 20 μL of 50 U/mL thrombin (1 U/mL final), and 625 μL of 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 μL of SFEGM-2, 100 μL of FBS (10% final), 100 μL of 5X Dulbecco's Modified Eagle Medium (DMEM (10% Final), 12 μL of 87.5 mM glyoxal (1 mM Final, Sigma) for crosslinking collagen, 50 μL of 0.1 N NaOH to neutralize acidic collagen, 20 μL of 50 U/mL thrombin (1 U/mL final), 250 μL of collagen stock solution (1.0 mg/mL final), and 375 μL of 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