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Effect of prevascularization on *in vivo* vascularization of poly(propylene fumarate)/fibrin scaffolds

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ABSTRACT

The importance of vascularization in the field of bone tissue engineering has been established by previous studies. The present work proposes a novel poly(propylene fumarate) (PPF)/fibrin composite scaffold for the development of vascularized neobone tissue. The effect of prevascularization (i.e., *in vitro* pre-culture prior to implantation) with human mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (HUVECs) on *in vivo* vascularization of scaffolds was determined. Five conditions were studied: no pre-culture (NP), 1 week pre-culture (1P), 2 week pre-culture (2P), 3 week pre-culture (3P), and scaffolds without cells (control, C). Scaffolds were implanted subcutaneously in a severe combined immunodeficiency (SCID) mouse model for 9 days. During *in vitro* studies, CD31 staining showed a significant increase in vascular network area over 3 weeks of culture. Vascular density was significantly higher *in vivo* when comparing the NP and 3P groups. Immunohistochemical staining of human CD-31 expression indicated spreading of vascular networks with increasing pre-culture time. These vascular networks were perfused with mouse blood indicated by perfused lectin staining in human CD-31 positive vessels. Our results demonstrate that *in vitro* prevascularization supports *in vivo* vascularization in PPF/fibrin scaffolds.

1. Introduction

The formation of strong bone requires a remodeling process that results in a highly organized array of osteocytes [1]. The mineralized extracellular matrix (collagen I and hydroxyapatite) surrounding these osteocytes includes a dense and profuse vascular supply [2]. The vascular supply of bone is required for providing oxygen and nutrients and acts as a conduit for new osteoprogenitor cells needed to maintain the newly formed bone [3]. Engineering functional bone requires the generation of an extensive microvascular network within the resultant tissue.

Bone tissue engineering aims to regenerate bone tissue through the development and application of bone tissue engineered grafts. These grafts are expected to be functionally active at the defect site and support the healing process, and, thereafter resorb at a rate compatible with the remodeling of the newly formed bone. Tissue engineered bone grafts typically consist of resorbable scaffolds prepared from polymeric, ceramic, and composite biomaterials along with cells and growth factors [4]. As yet, other than the Infuse (Medtronic, Minneapolis, MN) product, none has proved sufficient for healing critical-size or larger bone defects (i.e., 2–3 cm or greater) [5]. It is often because bone grafts of such sizes do not receive adequate supply of oxygen and nutrients at the inner regions of the graft, a necrotic core forms defeating the researcher's regenerative bone strategy. Therefore, it is critically important to establish a vascular supply throughout a graft, especially in large bone grafts, for successful bone regeneration to take place [6,7].

With an increase in the understanding of bone healing, the number of studies acknowledging the importance of vascularization in bone healing has increased [8,9]. Three frequently explored *in vitro* strategies for establishing a vascular supply in bone tissue

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engineered scaffolds are: 1) stimulation of vascularization from surrounding tissue via incorporation of growth factors or other proangiogenic stimuli [10], 2) bioreactor culture, and/or, 3) co-culture of endothelial cells along with mesenchymal stem cells or osteoblasts [11]. Bioreactors provide an artificial proxy for vascularization via continuous in-flux of nutrients and oxygen and out-flux of waste materials through the bioreactor system [12–17]. On the other hand, co-culture with endothelial cells (e.g., model cell types such as HUVECs or endothelial progenitor cells [EPCs]), establishes natural vascularization within the scaffold via the formation of blood vessels [18,19].

Another important method for establishing vascularization of a three dimensional scaffold is *in vivo* vascularization. This can be carried out in two possible ways: 1) Extrinsic vascularization via implantation and incubation of the scaffold beneath a highly vascularized region of the body such as subcutaneous tissue for a certain interval of time before implantation at the defect area, and, 2) Intrinsic vascularization though implantation of a vascular network into the scaffold which is further anastomized with the surrounding host vasculature through surgical intervention [48].

Co-culture of endothelial cells along with osteoblasts has been found to be favorable for bone formation and vascularization [20–23]. hMSCs and endothelial cell co-culture has been studied for the interplay and interaction of the two cell types for promoting osteogenic differentiation and vascularization *in vitro* [24]. These cells can be incorporated by co-seeding on the biomaterial scaffolds or culturing them in the form of three-dimensional spheroids [25]. Spheroid co-culture is advantageous in comparison with direct seeding of bone and blood vessel progenitor cells because it provides an environment similar to that of the body, where tissues are made up of more than one kind of cell arranged in 3 dimensional configurations [26]. Synergistic/co-stimulatory effects of hMSC/HUVEC spheroid co-culture have been studied. HUVECs not only form vascular networks but also promote osteogenesis and proliferation of hMSCs [27–30]. The hMSCs lead to bone formation while acting as trophic mediators for endothelial cells by secreting proangiogenic factors and acting as a scaffold for the formation and stabilization of vascular units [31,32].

Despite the importance of vascularization in bone tissue engineering, there is still a need to improve upon current methods for the prevascularization of bone tissue engineering scaffolds. Combining these prevascularization strategies with resorbable scaffolding biomaterials could ensure the appropriate size and shape of the forming bone [31]. Poly(propylene fumarate) polymer is a photocrosslinkable, biocompatible and resorbable biomaterial which can be 3-D printed into almost any desirable shape and size and can be rendered with high mechanical strength [33,34]. PPF has also been extensively studied for bone tissue engineering applications [33,35–38], though, the vascularization of these scaffolds is less studied [39–43]. Fibrin, which is formed naturally in the body during wound healing [44], is widely used to enhance vascularization due to its pro-angiogenic properties. However, its mechanical properties are less suitable for bone tissue engineering applications.

A recent study from our group evaluated the vascularization potential of PPF scaffolds through modeling and experimental studies based on 3D printed structure [34]. Incorporation of fibrin polymers into PPF as a composite system that combines the favorable properties of both the polymers for vascularization of bone tissue engineered scaffolds as well as to maintain the space of the defect have not been studied to our knowledge. We speculate that a composite scaffold of PPF and fibrin could provide a mechanically stable biomaterial system that facilitates vascularization (Fig. 1). Therefore, we developed a novel composite scaffold system of fibrin and a 3-D printed poly(propylene fumarate) shell in which we can study the effect of hMSC/HUVEC spheroid pre-culture on the vascularization of these scaffolds for bone tissue engineering applications. In the present study, our overall aim is to study the effect of *in vitro* spheroid pre-culture/prevascularization on *in vivo* vascularization response inside this clinically scalable scaffold system.

2. Methods

2.1. Experimental design

Each scaffold sleeve consisted of a fibrin hydrogel containing a single HUVEC/hMSC spheroid. Experiments were conducted in an organized manner. For the *in vitro* experiments, scaffolds were cultured for 1, 2, or 3 weeks ($n = 3$ per time point) prior to formalin fixing. Samples were stained for human CD-31 and α-SMA and imaged using confocal microscopy. The *in vivo* experiments involved five experimental groups with five mice each receiving four scaffolds subcutaneously. Therefore, each group consisted of twenty scaffolds. On the basis of the time of *in vitro* pre-culture of the fibrin-embedded spheroid before implantation, the experimental groups were as follows: 1) No pre-culture (NP) group, 2) 1 week pre-culture (1P) group, 3) 2 week pre-culture (2P) group, 4) 3 week pre-culture (3P) group, and 5) control (C) group (i.e., a solid-cured, resorbable polymeric sleeve containing fibrin without a spheroid). All groups were implanted for 9 days immediately following the group's respective pre-culture time. Mice from each group were sacrificed at the corresponding time point and a formaldehyde perfusion fixation procedure was undertaken. Out of the twenty scaffolds obtained per group, 8 were used for lectin imaging while 12 scaffolds were used for other histological analysis. Out of the 8 lectin imaged scaffolds, 3 were used for overall *in vivo* fluorescence imaging before preparing them for microscopic analysis.

2.2. Synthesis and sterilization of scaffold sleeves

Poly(propylene fumarate) (PPF) (Molecular weight = 1269 Da, PDI = 1.5) cylindrical sleeves were printed using an EnvisionTEC (Dearborn, MI) Perfactory 3 Standard 3D Printer. The resin chemistry involved is 1:1 PPF:DEF, 0.7% w/w Irgacure 819/BAPO, 0.4% w/w Oxybenzone/2-Hydroxy-4-methoxybenzophenone and 0.3% w/w Irgacure 784. Hollow, cylindrical scaffolds with dimensions of 6 mm height, 3 mm outer diameter, 0.25 mm wall thickness, and 0.35 mm pore size were built (Fig. 1). The scaffolds were washed in a sterile environment to remove any uncured resin using the following sequence: 15 min PBS, 30 min 70% acetone, three times each of 15 min PBS, 20 min 70% acetone, three times each of 10 min 70% acetone, two times each of 15 min PBS. The scaffolds were placed in fresh PBS and incubated at 37 °C in a petri dish for 72 h following by soaking in FBS for two nights. The washed scaffolds were placed upright on a petri dish and incubated at 37 °C until dry.

2.3. Cell culture and spheroid preparation

HUVECs were purchased at passage P1, cultured to confluency in EGM-2 media and seeded into spheroids at passage P4. hMSCs were cultured to confluency in T-175 flasks using hMSC differentiation basal medium and seeded at passage P5 into spheroids. Both HUVECs and hMSCs were obtained under an exemption from the IRB since they were purchased and were received de-identified from the vendor. Spheroids of 2500 HUVECs and 2500 hMSCs were created by suspending cells in 150 μL EGM-2 containing 0.25% w/v methylcellulose. Spheroids were incubated overnight in