



Effects of hydroxyapatite on endothelial network formation in collagen/fibrin composite hydrogels in vitro and in vivo



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ABSTRACT

Co-culture of endothelial cells (EC) and mesenchymal stem cells (MSC) results in robust vascular network formation in constrained 3-D collagen/fibrin (COL/FIB) composite hydrogels. However, the ability to form endothelial networks is lost when such gels are allowed to compact via cell-mediated remodeling. In this study, we created co-cultures of human EC and human MSC in both constrained and unconstrained COL/FIB matrices and systematically added nanoparticulate hydroxyapatite (HA, 0–20 mg ml⁻¹), a bone-like mineral that has been shown to have pro-vasculogenic effects. Constructs cultured for 7 days were assayed for gel compaction, vascular network formation, and mechanical properties. In vitro, robust endothelial network formation was observed in constrained COL/FIB constructs without HA, but this response was significantly inhibited by addition of 5, 10, or 20 mg ml⁻¹ HA. In unconstrained matrices, network formation was abolished in pure COL/FIB constructs but was rescued by 1.25 or 2.5 mg ml⁻¹ HA, while higher levels again inhibited vasculogenesis. HA inhibited gel compaction in a dose-dependent manner, which was not correlated to endothelial network formation. HA affected initial stiffness of the gels, but gel remodeling abrogated this effect. Subcutaneous implantation of COL/FIB with 0, 2.5 or 20 mg ml⁻¹ HA in the mouse resulted in increased perfusion at the implant site, with no significant differences between materials. Histology at day 7 showed both host and human CD31-stained vasculature infiltrating the implants. These findings are relevant to the design of materials and scaffolds for orthopedic tissue engineering, where both vasculogenesis and formation of a mineral phase are required for regeneration.

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1. Introduction

Transplantation and engraftment of engineered tissues requires creation of a vascular supply, either through vasculogenesis (the de novo formation of blood vessels) or through angiogenesis (the creation of new vessels via sprouting from existing vasculature) [1]. The diffusive limit for nutrient transport in most tissues has been suggested to be only a few hundred microns, so a new vascular supply to implanted tissue must be created to provide convective transport to the region [2]. A variety of model systems have been created to study the process of vasculogenesis, including 3-D systems using extracellular matrix proteins such as collagen, fibrin and Matrigel® [3–6]. Previous work in our laboratory has shown composite collagen/fibrin (COL/FIB) matrices to be permissive to endothelial network formation in vitro when human umbilical vein endothelial cells (EC) are co-cultured with bone-marrow-derived

mesenchymal stem cells (MSC) [7]. The degree of vasculogenesis was shown to be dependent on the EC:MSC ratio and the composition of the matrix.

In most studies of vasculogenesis in 3-D hydrogels in vitro, volume reduction of the matrix is prevented by constraining the sample at its boundaries. This technique prevents remodeling and compaction of the matrix by the contractile forces exerted by embedded cells. Unconstrained gel compaction leads to increased matrix density and a concomitant increase in matrix stiffness [8,9]. It has been suggested that matrix mechanics play an important role in regulating endothelial network formation. Increased stiffness has been shown to promote angiogenesis in some studies [10,11], while other studies have shown an inverse relationship between increased matrix stiffness and neovessel growth, both in vitro and in vivo [3,7,12–15]. In addition, unconstrained gel compaction has been shown to result in the regression of endothelial networks in vitro [16,17].

Bioceramics have been included in vasculogenesis and angiogenesis models to promote neovessel growth both in vitro and

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in vivo for bone tissue engineering applications. Bioactive glasses are reactive materials composed of glass–ceramic composites that have been shown to induce mineralization. These materials have also been shown to be proangiogenic at low concentrations, presumably by increasing endothelial cell proliferation via dissolution into ionic components [18–22]. Similarly, hydroxyapatite (HA) is the mineral component of bone, and has also been examined for its ability to promote both vasculogenesis and angiogenesis. Low concentrations of HA have been shown to be compatible with EC, and to maintain the prototypical morphology and biochemical markers associated with normal EC function [23,24]. HA has also been incorporated into 3-D silk scaffolds designed to promote angiogenesis [25], and it has been observed that production of vascular endothelial growth factor (VEGF) from MSC is increased on poly(lactide-co-glycolide)–HA composite scaffolds [26]. In addition to its proangiogenic biochemical effects, it has been suggested that HA can inhibit cell-mediated compaction of protein hydrogels by providing structural integrity to the extracellular matrix [27].

In the current study, we examined the addition of HA to COL/FIB composite hydrogels as a means to modulate the degree of vasculogenesis by seeded EC and MSC in both constrained and unconstrained model systems. Our motivation was the observation that vasculogenesis is inhibited in unconstrained 3-D hydrogels due to matrix compaction, but that HA can both have proangiogenic effects and reduce gel remodeling. We systematically added HA to 3-D composite hydrogels and examined vascular network formation in vitro. We also measured matrix compaction and the mechanical properties of the hydrogels in an effort to understand the relationship between construct morphology and vasculogenic response. Cell-seeded COL/FIB/HA constructs were then implanted subcutaneously into mice to determine whether the effects of HA translated to changes in neovascularization in vivo. These studies demonstrate the use of HA in protein-based composite matrices, and contribute to our understanding of how vasculogenesis can be modulated in bone tissue engineering applications.

2. Materials and methods

2.1. Cell culture

Human umbilical vein EC were harvested from umbilical cords as previously described [7]. Briefly, umbilical veins were irrigated with sterile phosphate-buffered saline (PBS) and then incubated with 0.1% collagenase (Type I; Worthington Biochemical, Lakewood, NJ, USA) at 37 °C for 20 min. The digestion product was collected, the vein was washed with PBS, and the resulting suspension was centrifuged. The cell pellet was re-suspended in Endothelial Growth Medium-2 (EGM-2; Lonza Inc., Walkersville, MD, USA) and plated into flasks. After 24 h, the cells were washed with PBS to remove residual erythrocytes. EC were cultured in EGM-2 and used at passage 4. Human bone marrow-derived MSC (Lonza) were cultured in Dulbecco's modified Eagle's medium – low glucose (DMEM; Thermo Scientific; Logan, UT) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin and streptomycin (Invitrogen), and used at passage 7. The medium was changed every other day for both cells types.

2.2. Formation of three-dimensional collagen/fibrin/hydroxyapatite hydrogels

COL/FIB composite hydrogels were created as previously described [7]. Briefly, bovine skin COL Type I (4.0 mg ml⁻¹; MP Biomedicals, Solon, OH, USA) was dissolved in 0.02 N acetic acid and bovine fibrinogen (4.0 mg ml⁻¹; Sigma Aldrich, St. Louis, MO, USA) was dissolved in EGM-2. Stock solutions of nanograde HA

(Sigma) with a particle size of <200 nm were prepared at concentrations of 0, 12.5, 25, 50, 100 and 200 mg ml⁻¹ HA in DMEM and sonicated for 1 h prior to use to facilitate homogeneous dispersion in hydrogels [28]. The stock HA solutions were autoclaved at 120 °C for 20 min prior to use to maintain sterility. Hydrogels were formed by adding COL and FIB at a mass ratio of 40/60 (total protein concentration 2.5 mg ml⁻¹) to a mixture of 10% FBS, 10% 5×-concentrated DMEM, 5% 0.1 N NaOH, 2% thrombin (0.1 UT ml⁻¹; Sigma) and 10% HA in DMEM at 4 °C. The resulting final concentrations of HA in the hydrogels were 0, 1.25, 2.5, 5, 10 and 20 mg ml⁻¹. Matrix mixtures (500 μl) were placed into a 24-well plate and gelled at 37 °C for 45 min. Both EC and MSC were added directly into the gel mixture to at a ratio of 1:1 EC:MSC, with a total cell concentration of 2.4 × 10⁵ cells ml⁻¹. Cells were added prior to gelation to facilitate the homogeneous distribution of cells throughout the hydrogels. For constrained culture studies, hydrogels were kept in the original 24-well plates and adhered to the walls of the wells. For unconstrained studies, constructs were freed from the well walls immediately after the 45 min incubation period and transferred to non-tissue-culture-treated 6-well plates. All hydrogels were cultured in EGM-2 for 7 days at 37 °C and 5% CO₂.

2.3. Endothelial network formation assay

Vessel-like structure formation was quantified as previously described [7]. Briefly, EC were labeled through a retroviral expression system (Orbigen Inc., San Diego, CA, USA) to enable stable expression of a fluorescent protein (mCherry; Clontech, Mountain View, CA, USA). Cell-seeded hydrogels were imaged at day 7 with a fluorescent microscope (Olympus America Inc., Center Valley, PA, USA). For both constrained and unconstrained hydrogels, five representative images were taken of each gel and analyzed using the Angiogenesis Tube Module in Metamorph Premier Software (Molecular Devices Inc., Sunnyvale, CA, USA). The total network length of vessel-like structures formed in vitro was calculated by setting a minimum width, maximum width and intensity over background.

2.4. Mechanical properties testing

Gel rheology was performed on acellular COL/FIB/HA hydrogels as previously described [7]. Briefly, pre-formed COL/FIB/HA solutions were loaded into a gel rheometer (AR-G2, TA Instruments, New Castle, DE, USA) and a time sweep was conducted for 45 min at 37 °C. The storage (*G'*) and loss (*G''*) moduli were calculated from the final 5 min of the time sweep.

Compressive testing was performed by placing hydrogels under uniaxial compression using a 1.5 mm hemispherical indenter mounted on a 50 g load cell in a Test Resources frame (Test Resources Inc., Shakopee, MN, USA), and therefore the system was capable of recording the entire force–displacement curve. Samples were removed from buffer and mounted on a dry rubber block to prevent slipping. Each was compressed at a rate of 0.33 cm s⁻¹ and force–displacement curves were generated at a sample rate of 200 Hz. Force–displacement curves were truncated to less than 25% compression and the Young's modulus (*E*) was determined from the equation below [29] using a non-linear least squares algorithm implemented in MATLAB.

$$E = \frac{3F(1-\nu^2)}{4r^{1/2}d^{3/2}}$$

where *E* is the compressive Young's modulus, *F* is the load measured by the load cell, *r* is the radius of the indenter, *ν* is the Poisson's ratio (assumed to be 0.5) and *d* is the depth of penetration measured by the linear encoder

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