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Influence of scaffold properties on the inter-relationship between human bone marrow derived stromal cells and endothelial cells in pro-osteogenic conditions

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ABSTRACT

One of the significant challenges in bone tissue engineering is the integration of biomaterials designed to facilitate and stimulate mineralization with a simultaneously rapid rate of angiogenesis and vascularization of the tissue construct, a challenge complicated by our lack of knowledge of the interactions among key cell types and scaffold properties.

This study compared functional activity of human bone marrow-derived stromal cells (hMSC) seeded onto a porous salt-leached poly(D,L-lactic acid) (PDLLA) scaffolds, with and without the incorporation of silk fibroin fibers and then further investigated their co-culture with human umbilical vein endothelial cells (HUVECs). Cell viability, proliferation, and alkaline phosphatase activity were measured for a range of time points in culture, with osteogenic and angiogenic marker immunolocalization and gene expression at selected stages.

Our findings suggest that, despite similar porosity and pore size distribution exhibited by the PDLLA and PDLLA plus silk fibroin scaffolds, there were marked differences in cell distribution and function. In the absence of fibers, a highly osteogenic response was observed in hMSCs in the scaffolds co-cultured with endothelial cells, greater than that observed with hMSCs alone or in either of the scaffolds with fibers added. However, fiber presence clearly better supported endothelial cell cultures, as determined by greater levels of endothelial marker expression at both the gene and protein level after 3 weeks of culture.

The design of composite scaffolds integrating beneficial components of differing structures and materials to facilitate appropriate biological responses appears a promising yet challenging avenue of research.

Statement of Significance

A significant challenge in bone tissue engineering is to promote a rapid vascularization of the tissue construct in parallel to the extracellular matrix mineralization. The design of composite scaffolds integrating beneficial components of differing structures and materials to facilitate appropriate biological responses appears a promising yet challenging avenue of research. Here we investigated cultures of hMSCs and HUVECs on a silk fibroin enhanced PDLLA scaffold, showing that the final output of this *in vitro* system is not the linear sum of the effects of the single variables. These results are of interest as they demonstrate how the addition of endothelial cells can affect hMSC phenotype and that the output can be further modulated by the introduction of silk fibroin fibers.

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1. Introduction

Tissue engineering research shows great promise for the treatment of tissue defects and bone non-union [1–3]. Nevertheless

major concerns such as the lack of vessel formation and scaffold integration have hindered translation of tissue engineering strategies to the clinic. While constructs of increasing complexity have been created, the roles of scaffold characteristics in the mineralization and vascularization processes of endogenous precursor cells remain unclear.

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Osteogenesis is a complex process that involves the well-orchestrated interaction of various cell types [4,5]. Vascularization is recognized to play a central role in normal bone tissue homeostasis and repair [6]. The processes integral to bone formation are thought to be dependent on angiogenesis due to the close interaction between the bone-forming osteoblasts (OBs) and the vessel-forming endothelial cells (ECs) [7–10].

Several *in vitro* studies have investigated the interaction between OBs and ECs using co-culture systems as tools for mimicking the complex structures and regulatory processes in living tissues. Proximity between OBs and ECs is necessary to establish a suitable model for studying the interaction between these two cell types, with both direct cell contact and paracrine mechanisms playing a role [7,8,11–13].

Consideration of pre-established culture systems is complex, given the use of multiple osteoprogenitor and osteoblast cell lines and endothelial cell types, and often leads to contradictory results. Nevertheless *in vitro* studies have shown that endothelial cells regulate the function of osteoprogenitor cells and vice versa [14–17]. While most authors have found that endothelial cells exert a positive effect on osteoprogenitor cell proliferation [14,16–18], their influence on osteoprogenitor cell differentiation is somewhat controversial [7,16,19,20]. We are particularly interested in the interaction of human bone marrow-derived stromal cells (hMSCs) with ECs. For this study we have selected the well-studied human umbilical vein endothelial cells (HUVECs). Although considerable research has been devoted to their involvement in angiogenesis, rather less attention has focussed on the relationship between hMSCs and ECs, and osteogenesis. It would also be of interest to learn how scaffold properties can influence the co-culture of hMSCs and HUVECs, and subsequent mineralization.

Silk fibroin [21–23] is a biomaterial that supports the attachment and growth of osteoblasts [24] and maintains a differentiated and functional endothelial phenotype in optimal medium conditions [25]. In particular, silk fibroin fibers seeded with a co-culture of endothelial cells and osteoblasts provides a natural structure with capillary-like formations if cultured in specific endothelial cell growth medium [11,26]. Unfortunately, scaffolds made with silk fibroin fibers to form a 3D fibrous scaffold usually lack porosity, leading to poor cell migration into the structures. To overcome this problem, we used a previously developed multi-component scaffold composed of a synthetic porous scaffold and a silk fibroin fibrous structure. The baseline scaffold was made using poly(D,L-lactic acid) (PDLLA), designed to promote mineralization [27,28], while silk fibroin was incorporated as the fibrous structure to trigger endothelial cell activity [29].

Here we examined the influence of scaffold properties on hMSCs and on the inter-relationship between HUVECs and hMSCs in osteogenic culture conditions, in terms of hMSC differentiation, HUVEC fate, and scaffold mineralization. To our knowledge, this is the first study that investigates cultures of hMSCs and HUVECs on a silk fibroin enhanced PDLLA scaffold.

2. Materials and methods

All chemicals were from Sigma–Aldrich (St. Louis, MO) unless otherwise specified.

2.1. Scaffold production

Two scaffolds were established as described previously. Group1 was a salt-leached PDLLA sponge and Group2 was obtained by adding silk fibroin fibers to Group1.

Group1 was produced by the solvent casting particulate leaching technique [30]. Briefly, PDLLA (Resomer R207S, Boehringer Ingelheim, Germany) was dissolved in dichloromethane:dimethyl

formamide (70:30 v/v) and a 7% (w/v) solution was prepared. Sieved NaCl particulates, ranging from 425 to 1180 μm , were mixed with PDLLA, obtaining a 1:25 w/w polymer/porogen final concentration. The mixture was air-dried (24–36 h) and immersed in deionized water for 3 days with changes every 6 h for the removal of the porogen.

Fibers were introduced into the scaffold as described previously [29]. The *Bombyx mori* silkworm cocoons were degummed, washed several times in distilled water and dried at room temperature. Then 0.05 g of these spread fibers was mixed with sieved NaCl particulates, ranging from 425 to 1180 μm as in Group1, and the PDLLA solution, obtaining a 1:14 w/w polymer/porogen and a 7.5/1 w/w PDLLA/SF final concentration. The mixture was air-dried and the porogen was removed as described above.

Before use, a biopsy punch was used to cut scaffolds with a diameter of 8 mm diameter. Scaffolds were sterilized with aqueous ethanol solution 70% (v/v) and dried under a sterile hood at room temperature.

2.2. *In vitro* cell culture

Bone marrow-derived human mesenchymal stromal cells (hMSCs), with established multipotency, were purchased from the Texas A&M Health Science Center, College of Medicine Institute for Regenerative Medicine (Temple, TX). Individual donor cell line was expanded to passage 3, using a starting density of 50 cells/cm² and cultures were maintained at under 80% confluency in Minimum Essential Medium alpha (α MEM) containing 16.7% fetal bovine serum (FBS; Atlanta Biologicals; Lawrenceville, GA) and 100 units/ml penicillin/100 $\mu\text{g}/\text{ml}$ streptomycin/2 mM L-glutamine (PSL; Invitrogen; Carlsbad, CA) at 37 °C and 5% CO₂. Stem cell phenotype was maintained by ensuring preservation of fusiform shape and adherence to cell culture dishes. After sterilization, scaffolds were placed in 48 well plates and washed with PBS. Each sample was seeded with 10 μl media containing 75,000 hMSCs or 75,000 hMSC plus 75,000 HUVECs (CC-2517, Lonza, Walkersville, MD) expanded to passage 4. After 24 h, scaffolds were moved to 24 well plates. All scaffolds were cultured at 37 °C, 5% CO₂ in osteogenic differentiation medium: MEM alpha medium (Life Technologies, Grand Island, NY); 16% (v/v) fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA); 100 U Penicillin, 100 μg Streptomycin, 2 mM L-glutamine (Life Technologies); 6 mM β -glycerol phosphate; 50 $\mu\text{g}/\text{ml}$ ascorbic acid 2-phosphate; 1 nM dexamethasone and 50 ng/mL L-thyroxine. Media were replaced every 48 h for a total of 6 weeks. Scaffolds were harvested at different experimental time points.

2.3. Proliferation

Cell number over time in culture was determined using a PicoGreen DNA quantification assay (Quant-iT PicoGreen dsDNA Assay Kit, Life Technologies). At pre-determined time points, media were removed from wells and scaffolds washed three times with PBS. 500 μl of 0.05% Triton-X/PBS was used to completely submerge the scaffold before freezing and storing at –20 °C until analysis. Each thawed sample was then sonicated for 10 s using a Virsonic ultrasonic cell disrupter (Virtis, Warminster, PA) and centrifuged. Samples (100 μl , in triplicate) were aliquoted into a black 96 well plate and 100 μl PicoGreen working solution added to each well. After 5 min the plate was read on a Perkin Elmer HTS 7000 fluorescent plate reader (excitation wavelength: 485 nm, emission wavelength: 535 nm). To calculate DNA concentrations, a calibration curve was generated using the double stranded lambda DNA standard provided with the kit. Results were divided by the average amount of DNA in a cell [31], to obtain estimates of cell number.

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