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Response of bone marrow stromal cells to graded co-electrospun scaffolds and its implications for engineering the ligament-bone interface

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ABSTRACT

Biomaterial scaffolds with gradients in architecture, mechanical and chemical properties have the potential to improve the osseointegration of ligament grafts by recapitulating phenotypic gradients that exist at the natural ligament-bone (L-B) interface. Towards the larger goal of regenerating the L-B interface, this *in vitro* study was performed to investigate the potential of two scaffolds with mineral gradients in promoting a spatial gradient of osteoblastic differentiation. Specifically, the first graded scaffold was fabricated by co-electrospinning two polymer solutions (one doped with nano-hydroxyapatite particles) from offset spinnerets, while the second was created by immersing the first scaffold in a 5 × simulated body fluid. Rat bone marrow stromal cells, cultured in the presence of osteogenic supplements, were found to be metabolically active on all regions of both scaffolds after 1 and 7 days of culture. Gene expression of bone morphogenic protein-2 and osteopontin was elevated on mineral-containing regions as compared to regions without mineral, while the expression of alkaline phosphatase mRNA revealed the opposite trend. Finally, the presence of osteopontin and bone sialoprotein confirmed osteoblastic phenotypic maturation by day 28. This study indicates that co-electrospun scaffolds with gradients in mineral content can guide the formation of phenotypic gradients and may thus promote the regeneration of the L-B interface.

1. Introduction

Interfacial tissue engineering is a rapidly growing field that aims to regenerate tissue interfaces through the use of graded scaffolds that recapitulate *in vivo* transitions between tissues [1]. In recent years, regeneration of the ligament-bone (L-B) interface has received significant attention due to its potential for improving the osseointegration of ligament grafts [2]. The L-B interface consists of complex gradients that transition over four different regions: bone, mineralized fibrocartilage, unmineralized fibrocartilage and ligament [3]. Since this interface consists of gradients in architecture, mechanical properties, chemical properties and cell phenotypes (Fig. 1), scaffolds possessing structural, mechanical and biochemical gradients have the potential to facilitate the regeneration of this interface [4]. Specifically, gradient cues in such scaffolds can help guide the formation of tissues with phenotypic gradients, which in turn may aid the osseointegration of ligament grafts [2,4]. With the long-term goal of regenerating the L-B interface, this *in vitro* study evaluates the potential for graded scaffolds to guide the establishment of an osteoblastic gradient.

Continuously graded scaffolds for regenerating interfacial tissues can be fabricated by the process of electrospinning [1]. This approach offers the ability to create nano-fibrous scaffolds and tune their mechanical and chemical properties via simple *in situ* alterations such as the use of multiple spinnerets [5]. Furthermore, electrospun scaffolds can be modified post-fabrication by performing appropriate chemical treatments to change their surface chemistry [6]. In particular, electrospun scaffolds with mineral gradients have been investigated for establishing gradients of cell response towards potentially engineering the L-B interface [7,8]. Recently, Samavedi et al created graded co-electrospun scaffolds with a spatial gradient in nano-hydroxyapatite (nHAP) content and subsequently immersed these scaffolds into a simulated body fluid to develop a second mineral gradient [9]. Both scaffolds possessed continuous gradients in mechanical properties, chemical properties

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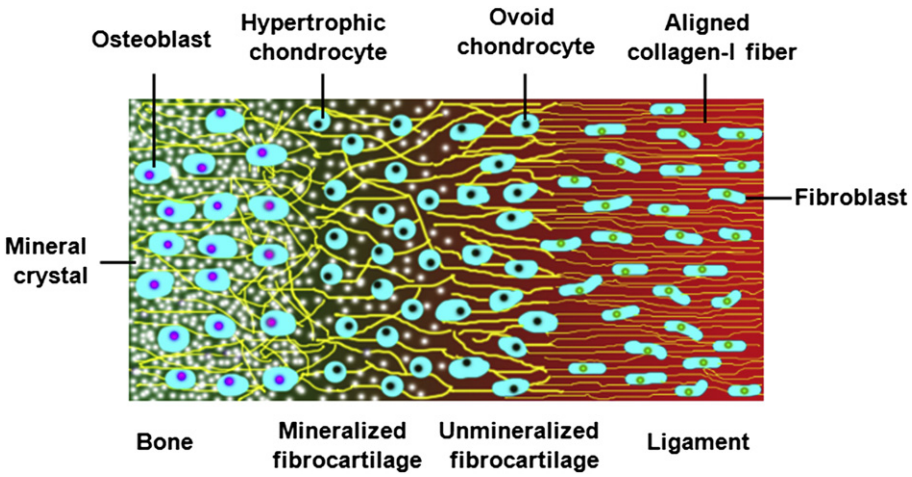

Fig. 1. Diagram of the L-B interface depicting gradients of mineral content, collagen fiber diameter/alignment and cell phenotype. The interface consists of complex gradients in mechanical and biochemical properties that smoothly transition over four regions: bone, mineralized fibrocartilage, unmineralized fibrocartilage and ligament.

and mineral content, and were found to support the growth of MC3T3-E1 osteo progenitor cells. However, the effect of mineral on cell attachment and osteoblastic differentiation was not tested in this study.

Incorporation of a mineral phase (e.g., HAP, tricalcium phosphate) into/onto scaffolds has been shown to influence mesenchymal stem cell behavior including attachment, osteoblastic differentiation and phenotypic maturation [10–13]. Osteoblastic differentiation is marked by the induction of proteins such as alkaline phosphatase (ALP), whose expression is elevated during early stages of differentiation, and growth factors such as bone morphogenetic protein-2 (BMP-2), whose early expression may initiate autocrine signaling pathways that result in maturation [14,15]. Osteoblastic phenotypic maturation is characterized by deposition of extracellular matrix (ECM) proteins like osteopontin (OPN) and bone sialoprotein (BSP).

The goal of this study was to characterize the role of scaffolds possessing mineral gradients in influencing osteoblastic differentiation and maturation of bone marrow derived stromal cells (BMSCs). Towards this end, scaffolds with two different mineral gradients were fabricated. The first scaffold was created by co-electrospinning nHAP-doped poly(caprolactone) (nHAP-PCL) and poly(ester urethane urea) (PUR) solutions from offset spinnerets to result in gradients of fiber chemistry and mineral content. The second scaffold was prepared by treating the co-electrospun graded scaffold with a 5 × simulated body fluid (5 × SBF). Here, nHAP particles were chosen for their stability and superior osteoconductive properties, compared to other types of ceramics [16,17]. Moreover, their inclusion into electrospun PCL fibers was intended to promote selective nucleation of mineral crystallites when treated with the 5 × SBF solution. Thereafter, rat BMSCs were cultured on scaffolds in the presence of osteogenic supplements. Subsequently, cell metabolic activity, morphology, density, mRNA expression for BMP-2, ALP, OPN and BSP, and deposition of OPN and BSP were investigated.

2. Materials and methods

2.1. Materials

Chemicals and laboratory supplies were purchased from Fisher Scientific (Pittsburgh, PA), while biological supplies were purchased from Life Technologies (Gaithersburg, MD), unless otherwise noted. PCL (inherent viscosity: 1.15 dL/g in chloroform) was purchased from LACTEL biodegradable polymers (Birmingham, AL), while a linear segmented degradable PUR was synthesized from 2 kDa PCL diol as described previously [18]. Sodium sulfate salt and 2,2,2- trifluoroethanol (TFE) were purchased from Acros Organics (Morris Plains, NJ). Alizarin red S dye, nHAP particles (<200 nm), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), trypsin/sodium salt of ethylenediamine tetraacetic acid (trypsin/EDTA), dexamethasone, disodium salt of glycerol-2-phosphate, ascorbic acid, Triton X-100, rhodamine-phalloidin, 4′,6-diamidino-2-phenylindole (DAPI) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Calabasas, CA), while phenol red-free Eagle's-MEM and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from MP Biomedicals (Solon, OH). RNeasy Mini kit, DNase I and QIAshredder columns were purchased from Qiagen (Valencia, CA), methanol-free formaldehyde from Polysciences Inc (Warrington, PA) and primers from Integrated DNA Technologies (Coralville, IA). Primary antibodies against OPN and BSP, and fluorescein thioisocyanate (FITC)-conjugated secondary antibody were purchased from Abcam Inc. (Cambridge, MA). Vectamount AQ was purchased from Vector labs (Burlingame, CA).

2.2. Fabrication of graded scaffolds

Scaffolds with gradients in chemistry and mineral content were fabricated by co-electrospinning two polymer solutions from offset spinnerets, as described previously [9]. Briefly, a 14% (w/v) PCL solution containing 3.5% (w/v) nHAP particles was prepared in TFE, while PUR was dissolved in HFIP at 12% (w/w). These concentrations were selected because they resulted in similar fiber diameters: ~0.55 μm and ~1.65 μm for the PCL region (bimodal diameter distribution), and ~1.65 μm for the PUR region [9]. These two solutions were loaded into separate 10 mL plastic syringes and initially co-electrospun onto a single region of a grounded rotating mandrel wrapped in aluminum foil. Subsequently, the syringes were offset ~7 cm along the length of the mandrel to achieve a gradient in fiber deposition and mineral content. Electrospinning was performed for over an hour and the resultant graded scaffold, denoted ES scaffold (for electrospun scaffold), was stained with Alizarin Red S dye to demonstrate the gradient. Briefly, the scaffold was immersed in a 40 mM solution of dye in de-ionized water for 2.5 h, followed by thorough washing in de-ionized water. Images of the scaffold were captured after air drying.

After electrospinning, the ES scaffold was divided into three regions for ease of processing and characterization: a region consisting only of nHAP-doped PCL fibers (nHAP-PCL(ES)), a region consisting solely of PUR fibers (PUR(ES)) and a transition region consisting of a mixture of both types of fibers (GRAD(ES)). Subsequently, samples from various regions of the ES scaffold were treated with approximately 40 mL of a 5 × SBF solution, prepared using a previously reported formulation [9]. Samples were treated for 2 h at 37 °C with gentle agitation to coat the surface of the fibers with a calcium phosphate mineral layer (CaP). Previous analysis of a mineral layer produced in this manner suggested that it consists of calcium-deficient hydroxyapatite crystallites [9]. After treatment, the 5 × SBF solution was discarded and the samples were rinsed gently with de-ionized water and dried in a desiccator before further use. The resulting 5 × SBF-treated scaffold was designated SBF scaffold and its various regions were denoted nHAP-PCL(SBF), GRAD(SBF) and PUR(SBF), corresponding to similar regions in the ES scaffold.

2.3. Scanning electron microscopy (SEM)

nHAP particles and regions from both types of graded scaffolds were examined using SEM to visually estimate size range and surface coverage of mineral respectively. Briefly, samples were mounted onto SEM studs and sputter-coated with