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The effect of mesenchymal stem cells delivered via hydrogel-based tissue engineered periosteum on bone allograft healing

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ABSTRACT

Allografts remain the clinical "gold standard" for treatment of critical sized bone defects despite minimal engraftment and ~60% long-term failure rates. Therefore, the development of strategies to improve allograft healing and integration are necessary. The periosteum and its associated stem cell population, which are lacking in allografts, coordinate autograft healing. Herein we utilized hydrolytically degradable hydrogels to transplant and localize mesenchymal stem cells (MSCs) to allograft surfaces, creating a periosteum mimetic, termed a 'tissue engineered periosteum'. Our results demonstrated that this tissue engineering approach resulted in increased graft vascularization (~2.4-fold), endochondral bone formation (~2.8-fold), and biomechanical strength (1.8-fold), as compared to untreated allografts, over 16 weeks of healing. Despite this enhancement in healing, the process of endochondral ossification was delayed compared to autografts, requiring further modifications for this approach to be clinically acceptable. However, this bottom-up biomaterials approach, the engineered periosteum, can be augmented with alternative cell types, matrix cues, growth factors, and/or other small molecule drugs to expedite the process of ossification.

1. Introduction

Critical-sized bone defects are very prevalent and result from skeletal defects, traumatic injuries, and tumor resections [1]. Clinically, >500,000 and >2.2 million bone graft procedures are performed annually in the United States and worldwide, respectively [2,3]. Reliable and effective techniques for bone processing and infectious disease detection has resulted in allografts becoming the clinical "gold standard" for treatment of critical sized bone defects [3–5]. Unlike autografts, which can be harvested in small volumes from non-load bearing regions of the skeleton, processed cadaveric allografts are readily available and fill the need for large volumes of graft material [3]. Additionally, allografts obviate donor site pain and morbidity, complications often resulting from autograft harvesting [6], and are mechanically superior to alternatives such as morselized bone graft materials, providing a structural advantage *in vivo* [3]. Despite these attributes, allografts exhibit minimal engraftment and a 60%, 10-year post-implantation failure rate due to fibrotic nonunions (~17%), infections (~8%), and microcrack propagation resulting in secondary fractures (~35%) [7–9].

In contrast to allografts, autografts completely heal, orchestrated by the periosteum, a thin layer of tissue covering the outer surface of bone that is comprised of an inner osteogenic cambrial layer and an outer fibrous layer [4,5,10–15]. The cambrial layer houses committed osteoblasts, osteogenic precursors, and periosteal stem cells. The periosteal stem cell is critical for endochondral bone formation in cortical bone healing [16]. However, the role of these cells remains unclear, as they may contribute to healing through a myriad of functions, including proliferation, chondrogenic differentiation, and endochondral ossification, and/or through the release of paracrine signals resulting in recruitment and activation of host osteoprogenitor cells [4,5,12–15,17–22]. Nevertheless, recent literature has demonstrated that intact periosteal tissue, which is lacking in processed allografts, is vital towards vascularization, bone callus formation, and subsequent healing and remodeling of autografts in the context of critical sized defect repair [4,5,10–12]. Using a murine defect model wherein a 5 mm segment of femur was resected and replaced with an autologous bone graft, the periosteum has been found to account

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for greater than 70% of new bone formation during healing [5,23]. In addition, removal of the periosteum from autografts has been shown to result in a 63% reduction in new bone formation [4,5,10].

Many attempts to emulate the healing orchestrated by the periosteum have exploited cell and/or growth factor delivery [4,5,14,15,19,20,23–26]. Towards growth factor mediated healing, delivery of bone morphogenetic protein 2 (BMP-2) [4,5,22,26], basic fibroblast growth factor (FGF-2) [17,24], parathyroid hormone (PTH) [27] and its peptide fragment teriparatide (PTH_{1-34}) [28], and vascular endothelial growth factor (VEGF) have been most commonly employed [19,20,25,29]. While these approaches have resulted in variable outcomes with respect to allograft revitalization, to date none have matched the success of autograft healing. Furthermore, growth factor delivery is plagued by a host of complications including immunogenic concerns and diffusion and/or degradation of growth factors, which requires delivery of supraphysiologic concentrations, leading to costly clinical translation, and potential off-target pathway activation [20,27,30,31]. Therefore, Food and Drug Administration approval for such approaches remains a significant hurdle [31–33].

Delivery of cells alone or in combination with the aforementioned growth factors is a common approach to improve allograft remodeling. Mesenchymal stem cells (MSCs) are commonly employed as they have been shown to be similar to periosteum stem cells [16,18,34–38], can be easily harvested in a patient specific manner, and are non-immunogenic, making their utilization in both allogeneic and autogeneic applications feasible [37]. Numerous preclinical models have demonstrated MSC therapeutic efficacy and regenerative capacity in a variety of musculoskeletal tissues [4,39–41]. Towards emulation of periosteum function, direct delivery of MSCs in the absence of a biomaterial results in negligible improvements in allograft healing [4,5,21]. Without carriers, MSCs exhibit poor graft localization, extensive migration into surrounding tissue, and limited cell survival [4,5,21]. To overcome these complications, numerous biomaterials have been investigated as periosteum mimetics. These include naturally derived acellular matrices, such as dermis and intestinal mucosa [4,21], commercially available collagen-based sponges [5], and synthetic polymers such as poly(lactide-co-glycolide) [29]. While these materials have been shown to improve cell localization to allograft surfaces, they suffer from irreproducible cell seeding and inadequate cell survival [4,5,21]. In addition, common scaffolds are poorly hydrated and are not easily modified biochemically or biomechanically to mimic periosteum characteristics [29,41–44].

Use of cell transplantation, growth factor delivery, and combinations thereof have improved allograft healing. However, they fail to yield adequate chondrocyte differentiation and endochondral ossification within transplanted cell populations, resulting in insufficient healing as compared to autograft controls [4,5,19,21]. In contrast to traditional scaffold materials poly(ethylene glycol) (PEG) hydrogels emulate the mechanical properties and hydration of the native extracellular matrix environment, making them ideal for many tissue engineering applications [41,43,45–47]. In addition, PEG hydrogels are easily modified to allow for degradation and inclusion of biomolecules and other cell-adhesion ligands to promote specific cell function [41,43,45–47].

In this work, we developed PEG hydrogels, which were designed to have consistent hydration, elastic properties, and provide similar cellular persistence as the periosteum, to transplant and localize MSCs to allograft surfaces. The resulting tissue engineered (T.E.) periosteum only provided signals for cell survival, acting as a 'blank slate' to assess MSC-mediated allograft healing. Using live animal fluorescent imaging, micro-computed tomography analysis of vascular and bone callus volume, histological staining, and biomechanical testing, T.E. periosteum-mediated healing of allografts was compared to autografts and untreated allografts, respectively, over 16 weeks.

2. Materials and methods

All materials were purchased from Sigma–Aldrich unless otherwise specified.

2.1. Synthesis of poly(ethylene glycol) (PEG) macromolecular monomers (macromers)

2.1.1. Hydrolytically-degradable PEG macromers

Degradable, PEG-based tri-block copolymers [methacrylate-poly(lactide)-b-PEG-b-poly(lactide)-methacrylate] (PEGPLADM, Fig. S1A), were synthesized as previously described by functionalizing linear PEG (Alfa Aesar, MW 10 kDa, $n = 227$) with D,L-lactide and performing microwave-assisted methacrylation [48–50]. To determine the number of lactide units and methacrylate functional groups per PEG macromer ^{1}H NMR analysis was used (Bruker Avance 400 MHz, $CDCl_3$).

2.1.2. Synthesis of acrylate-PEG-RGDS

The cell-adhesive sequence Arg-Gly-Asp-Ser (RGDS; 433 Da, EMD Chemicals, San Diego CA) was coupled to acrylate-PEG-N-Hydroxysuccinimide (MW 3500 Da, Jenkem Technology, Beijing China) through the amino terminus, as previously described, and allowed for tethering into hydrogels [49]. The product (acrylate-PEG-RGDS, Fig. S1B) was dialyzed against deionized water (molecular weight cutoff = 1000 Da, Spectrum Labs, Rancho Dominguez CA), lyophilized, analyzed via matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF, Bruker AutoFlex III SmartBeam) (solvent: 50% acetonitrile in H_2O + 0.1% TFA; matrix: α-cyano-4-hydroxy cinnamic acid (TCI Europe); calibrant: Peptide Calibration Standard (Brucker)) (*m/z Na*+, 4070 Da), and stored at 4 °C.

2.2. Cell culture

Mouse MSCs expressing green fluorescent protein (GFP^+ mMSCs) isolated from GFP transgenic mice (C57BL/6-Tg(UBC-GFP)30Scha/J) were obtained from the mesenchymal stem cell distribution center at Texas A&M (passage 6) [51]. GFP^+ mMSCs were grown at 37 °C and 5% CO_2 in growth media consisting of Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% fetal bovine serum, 10% horse serum (Atlanta Biologicals, Lawrenceville, GA, USA), 100 units/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza), and 0.25 μg/ml amphotericin B (Lonza). GFP^+ mMSCs were used prior to passage 10.

2.3. Bone graft preparation and transplantation

2.3.1. Mouse strains

Female 6–8 week old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Allogeneic bone grafts for implantation into C57BL/6 mice were obtained from freshly euthanized, age-matched wild-type BALB/c mice received from various research groups within the University of Rochester Medical Center.

2.3.2. Murine segmental femoral graft model

In vivo healing of bone grafts was assessed using a previously established murine segmental femoral graft model [4,5,23]. Briefly, 6–8 week old C57BL/6 mice were anesthetized using a combination of ketamine and xylazine (60 mg/kg and 4 mg/kg, respectively) administered via intraperitoneal injections. An 8 mm long incision was made, and blunt dissection of muscle was used to expose the mid-shaft femur. A 5 mm mid-diaphyseal segment was removed from the femur using a Dremel with a diamond blade attachment. A 5 mm cortical bone graft (autografts, allografts, or T.E. periosteum modified allografts) was transplanted into the femur defect and stabilized using a 22-gauge intramedullary pin. For live bone autograft transplantation, the graft was carefully dissected without compromising the periosteum, and immediately transplanted back into the same mouse. For devitalized bone graft transplantation, the grafting procedure was performed between mice with genetically different backgrounds. Briefly, allografts were scraped to physically remove periosteal tissue, flushed repeatedly with phosphate buffered saline (PBS) to remove marrow, sterilized with 70% ethanol, rinsed in PBS to remove residual ethanol, and flash frozen at −80 °C for at least 1 week prior to transplantation. It should be noted that all animal surgery procedures were performed under protocols approved by University Committee of Animal Resources (UCAR).

2.3.3. Photoencapsulation of GFP^+ mMSCs in PEG hydrogels around decellularized allografts (e.g., tissue engineered periosteum)

A 10 wt% solution of PEGPLADM was prepared in PBS with 2.0 mM acrylate-PEG-RGDS to maintain MSC viability through integrin interactions [42,45,52,53]. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as previously described [54], and added at a final concentration of 0.05 wt%. Trypsinized MSCs were added to the PEG macromer solution to achieve a final concentration of 25 million cells/mL. As previously described [11], a custom mold was used to form T.E. periosteum modified allografts. Briefly, 20 μL of PEG/