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Emulating native periosteum cell population and subsequent paracrine factor production to promote tissue engineered periosteum-mediated allograft healing

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ABSTRACT

Emulating autograft healing within the context of decellularized bone allografts has immediate clinical applications in the treatment of critical-sized bone defects. The periosteum, a thin, osteogenic tissue that surrounds bone, houses a heterogenous population of stem cells and osteoprogenitors. There is evidence that periosteum-cell derived paracrine factors, specifically vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP2), orchestrate autograft healing through host cell recruitment and subsequent tissue elaboration. In previous work, we demonstrated that the use of poly(ethylene glycol) (PEG) hydrogels as a tissue engineered (T.E.) periosteum to localize mesenchymal stem cells (MSCs) to the surface of decellularized bone enhances allograft healing and integration. Herein, we utilize a mixed population of 50:50 MSCs and osteoprogenitor cells to better mimic native periosteum cell population and paracrine factor production to further promote allograft healing. This mixed cell population was localized to the surface of decellularized allografts within degradable hydrogels and shown to expedite allograft healing. Specifically, bone callus formation and biomechanical graft–host integration are increased as compared to unmodified allografts. These results demonstrate the dual importance of periosteum-mediated paracrine factors orchestrating host cell recruitment as well as new bone formation while developing clinically translatable strategies for allograft healing and integration.

1. Introduction

The importance of the periosteum in coordinating autograft healing and repair is well established [1–8]. The cells of the periosteum, particularly periosteal stem cells and osteoprogenitors directly contribute to initial callus production, as well as through paracrine activation and recruitment of host cells [5,8–10]. Using lineage-tracing techniques and murine femoral defect models, peak autograft callus formation occurs ~21 days post-implantation, during which time initial donor periosteum-mediated cartilaginous matrix production is replaced by host-derived mineralized tissue [10]. Furthermore, removal of the periosteum results in a 63% decrease in new bone formation during autograft healing and repair [10,11]. Similarly, decellularized bone allografts, the clinical "gold standard" of treatment for critical sized bone defects, exhibit a comparable 61% decrease in bone formation as compared to autografts [12]. As previously noted, removal of the periosteum, as is the case in decellularized allografts, not only results in the removal of critical stem cell and osteoprogenitor cell populations, but also the growth factors produced by these cells [1–8]. Following injury, bone lining cells, circulating platelets, and peripheral cell populations release growth factors, including bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet derived growth factor (PDGF), transforming growth factor-β (TFG-β), and vascular endothelial growth factor (VEGF) [1,5,8,9,13]. In particular, the cells of the periosteum have been shown to secrete large quantities of

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VEGF and BMP2 following injury [14–19]. In addition, MSCs, which are phenotypically comparable to periosteal stem cells, exhibit comparable paracrine factor expression [5,20].

VEGF and BMP2 play critical roles in initiating and regulating bone healing, and successful remodeling requires precise activation and temporal expression of these factors [13,21–25]. In the context of autografts, VEGF is critical in the early phases of healing and induces angiogenesis, vascular sprouting, and capillary permeability [8,26]. BMP2 plays a role in later healing events, regulating matrix mineralization and new bone formation through both intramembranous and endochondral ossification [13,23,27,28]. VEGF and BMP2 also act synergistically during early angiogenesis and matrix mineralization to promote cellular proliferation, callus formation, and generation of cell populations necessary for endochondral ossification [25,26,29–31]. In an effort to emulate autograft healing in the context of decellularized bone allografts, numerous strategies have investigated growth factor delivery strategies [10–12,32–37]. In particular, dual delivery of VEGF and BMPs has been commonly employed to synergistically enhance bone production with variable success [10–12,32–40]. However, exogenous administration of growth factors is complicated by diffusion and degradation, as well as the supraphysiological doses required for biological effects that may lead to off-target pathway activation [23,41,42]. As an example, Cao et al. demonstrated enhanced repair within a rabbit segmental defect model via transplantation MSCs overexpressing ANG1 [43].

To overcome complications associated with the delivery of exogenous growth factors, some approaches have focused on transplantation or recruitment of cells to promoted allograft revitalization by producing cues for healing and repair [5,8,12,13,21,36,44]. Long et al. augmented decellularized allografts with MSC cell sheets and demonstrated significant enhancements in callus bone formation and allograft healing and integration 6 weeks post-implantation [36]. The healing was attributed to transplanted MSCs, which are known to secrete myriad soluble factors that are critical for healing, including, but not limited to VEGF, BMP2, angiopoietin 1 (ANG1), and stromal-derived factor-1 (SDF1) [1,13,21,45–48]. To better mimic native autograft cell populations and subsequent paracrine factor production, *ex vivo* MSC differentiation strategies have also been utilized prior to MSC transplantation [1,13,33]. For example, Ma et al. transplanted osteoblasts derived from MSCs to enhance bone formation within a rabbit mandible defect [33]. Similarly, transplantation of osteoblasts to critical sized calvarial defects were able to activate host MSCs, resulting in enhanced bone formation and healing [49]. Although these studies did not longitudinally track transplanted cell, the data suggests that *ex vivo* phenotypic modulation of transplanted cells enhances healing, possibly through modified paracrine factor production.

Previously, we demonstrated that PEG-based hydrogels can be used to localize MSCs to the surface of decellularized bone allografts [4,12]. T.E. periosteum-modified allografts result in significant increases in graft vascularization, bone callus formation, and biomechanics, as compared to unmodified allograft controls 16 weeks post-implantation in a segmental femoral defect model [12]. Despite the observed increases in healing, endochondral ossification was significantly delayed compared to autograft controls [12]. In an effort to expedite the rate of endochondral ossification, enhance the rate of allograft healing and integration, and further emulate the dual functionality of the native tissue, the T.E. periosteum transplanted cell population was modified to mimic the native periosteum cell population and subsequently native autograft paracrine factor production [2,3,5–7]. Towards this end, a subset of MSCs were differentiated into osteoprogenitor cells, combined with unaltered MSCs in a 50:50 mixture, and transplanted *in vivo* to create a T.E. periosteum to more closely emulate native periosteum-mediated healing observed in autografts [12,33].

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

Hydrolytically degradable, PEG-based tri-block copolymers PEGPLADM (methacrylate-poly(lactide)-b-PEG-b-poly(lactide)-methacrylate) (Fig. 1A), were synthesized by functionalizing linear PEG (Alfa Aesar, MW 10 kDa) with D,L-lactide and performing microwave-assisted methacrylation, as previously described [12,50–53]. ^{1}H NMR analysis (Bruker Avance 400 MHz, $CDCl_3$) was used to determine the number of lactide units (m = 3) and methacrylate functionality (>95%) per PEG macromer ($-CH_2CH_2O-$ (PEG), 908H, 3.2–3.8 ppm, multiplet; $-OCH(CH_3)COO-$, 4H/PLA repeat, 5.2–5.3 ppm, multiplet; $-OCH(CH_3)COO-$, 12H/PLA repeat, 1.4–1.6 ppm, multiplet; $CH_2{=}C(CH_3)-$, 4H/macromer, 5.6 and 6.3 ppm, singlets; $CH_2{=}C(CH_3)-$, 6H/macromer, 1.9 ppm, singlet).

2.1.2. Synthesis of acrylate-PEG-RGDS

The cell adhesive sequence Arg-Gly-Asp-Ser (Fig. 1; RGDS; 433 Da, EMD Chemicals, San Diego CA) was coupled to acrylate-PEG-N-Hydroxysuccinimide (Jenkem Technology, Beijing China, MW 3500 Da, p = 79) through the amino terminus to allow for incorporation into hydrogels, as previously described [12,51]. The product (Acrylate-PEG-RGDS, Fig. 1) 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 mass spectrometry (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 (Bruker, #206195)) (*m/z Cl*–, 3854 Da), and stored at 4 °C.

2.2. Cell culture

Bone marrow derived 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) [54]. 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 (FBS), 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). Where indicated, GFP^+ mMSCs were differentiated into osteoprogenitors via standard osteogenic induction media for a period of 10 days [33,55] (e.g., low-glucose Dulbecco's Modified Eagle Medium (DMEM, Thermo) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid-2-phosphate (2-phospho-L-ascorbic acid)). Osteogenic differentiation of MSCs was confirmed via gene expression analysis and histological staining [51]. 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 Taconic (Hudson, NY). Allogeneic bone grafts for implantation into C57BL/6 mice were dissected from freshly euthanized, age-matched wild-type BALB/c mice obtained 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 [10–12,56]. Briefly, 6–8 week old C57BL/6 mice were anesthetized via an intraperitoneal injection of ketamine and xylazine (60 mg/kg and 4 mg/kg, respectively). An 8 mm long incision was made, and blunt dissection was used to expose the mid-shaft of the femur. A Dremel with a diamond blade attachment was then used to remove a 5 mm mid-diaphyseal segment from the femur. 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 autograft transplantation, the graft was carefully dissected to maintain an intact periosteum, and immediately transplanted back into the same mouse. For devitalized bone allograft transplantation, the grafting procedure was performed between mice with genetically different backgrounds. Allografts were scraped to 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 stored at −80 °C for at least 1 week prior to transplantation. All animal surgery procedures were performed under protocols approved by The University of Rochester Committee of Animal Resources (UCAR).