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Bone regeneration using an alpha 2 beta 1 integrin-specific hydrogel as a BMP-2 delivery vehicle

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ABSTRACT

Non-healing bone defects present tremendous socioeconomic costs. Although successful in some clinical settings, bone morphogenetic protein (BMP) therapies require supraphysiological dose delivery for bone repair, raising treatment costs and risks of complications. We engineered a protease-degradable poly(-ethylene glycol) (PEG) synthetic hydrogel functionalized with a triple helical, α2β1 integrin-specific peptide (GFOGER) as a BMP-2 delivery vehicle. GFOGER-functionalized hydrogels lacking BMP-2 directed human stem cell differentiation and produced significant enhancements in bone repair within a critical-sized bone defect compared to RGD hydrogels or empty defects. GFOGER functionalization was crucial to the BMP-2-dependent healing response. Importantly, these engineered hydrogels outperformed the current clinical carrier in repairing non-healing bone defects at low BMP-2 doses. GFOGER hydrogels provided sustained *in vivo* release of encapsulated BMP-2, increased osteoprogenitor localization in the defect site, enhanced bone formation and induced defect bridging and mechanically robust healing at low BMP-2 doses which stimulated almost no bone regeneration when delivered from collagen sponges. These findings demonstrate that GFOGER hydrogels promote bone regeneration in challenging defects with low delivered BMP-2 doses and represent an effective delivery vehicle for protein therapeutics with translational potential.

1. Introduction

Over 1 million bone grafting, bone excision and fracture repair surgeries are performed annually in the US at a cost of approximately $5 billion [1–4]. While autografts are the gold-standard therapy for non-healing bone defects, these grafts are limited by low availability as well as donor site pain and inflammation [5]. More recently, bone morphogenetic protein (BMP) therapies have emerged as promising alternatives to autografts and allografts. While BMP therapy has been successful in stimulating bone repair, the BMP doses used clinically are orders of magnitude higher [6] than physiological concentrations of BMP, resulting in high costs of treatment and complications such as ectopic bone formation, nerve injuries and inflammation [5,7–10]. Therefore, there is an unmet clinical need for improved BMP delivery vehicles which promote bone healing at low delivered BMP doses to enable safe, cost-effective and efficacious BMP treatments.

Hydrogels, water-swollen cross-linked polymer networks, offer tremendous advantages as vehicles for protein delivery due to their high cytocompatibility, low inflammatory profile, tailorable mechanics and biofunctionality, and injectable delivery method [11–13]. In particular, poly(ethylene glycol) (PEG) hydrogels are attractive because they resist non-specific protein adsorption, providing a 'clean-slate' background onto which desired biofunctionalities may be incorporated [14]. In addition, PEGs are widely used in FDA-approved therapeutic products as covalent modifiers of proteins and lipids [15], indicating a history of safety in patients. This has led to increasing research interest in delivering protein therapeutics such as BMP-2 and BMP-7 from synthetic and natural hydrogels to improve bone healing [16–19].

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We have recently established PEG hydrogels cross-linked via maleimide groups as an alternative cross-linking chemistry to address limitations associated with other widely used PEG hydrogel polymerization chemistries such as free-radical polymerization [20]. The maleimide reactive group is extensively used in peptide bioconjugate chemistry because of its fast reaction kinetics and high specificity for thiols at physiological pH. Maleimide-based cross-linking of PEG hydrogels has significant advantages over other cross-linking chemistries, namely well-defined hydrogel structure, stoichiometric incorporation of bioligands, increased cytocompatibility, improved cross-linking efficiency, and reaction time scales appropriate for *in situ* gelation for *in vivo* applications [20]. Additionally, the base macromer exhibits minimal toxicity and inflammation *in vivo* and is rapidly excreted via the urine [21] – important considerations in establishing the safety and translational potential of these hydrogels.

A critical consideration in the design of protein delivery systems for regenerative medicine is the incorporation of extracellular matrix (ECM)-mimetic adhesive ligands. Many orthopedic biomaterials utilize ECM-inspired peptides which promote integrin–ECM interactions to direct desired host cell responses [16,22,23] as these interactions regulate cell survival, proliferation, migration and differentiation [24–26]. In particular, the interaction of α2β1 integrin with collagen I is a crucial signal for osteoblastic differentiation and mineralization [27–32]. The hexapeptide sequence Gly–Phe–Hyp–Gly–Glu–Arg (GFOGER), residues 502–507 of the α1(I) chain of type I collagen, serves as the major recognition site for α2β1 integrin binding [33–35]. Our group has previously engineered a synthetic collagen I-mimetic GFOGER-containing peptide, GGYGGGP$(GPP)_5$GFOGER$(GPP)_5$GPC, which recapitulates the triple helical structure of native collagen (Fig. S1) and binds α2β1 integrin with high affinity and specificity [36]. GFOGER peptide coatings on plastic, titanium and poly(caprolactone) support equivalent levels of α2β1 integrin-mediated cell adhesion as native collagen I [36], promote osteoblastic differentiation *in vitro* [22,37], improve fixation of metal implants to rat cortices [22], and enhance bone healing in rat femur defects [38]. In contrast to the collagen I-mimetic GFOGER peptide, the widely used bioadhesive RGD peptides bind primarily to the αvβ3 integrin and do not have intrinsic osteogenic properties [39–41].

We hypothesized that presentation of the pro-osteogenic α2β1 integrin-specific GFOGER peptide to host cells combined with sustained release of low doses of BMP-2 would direct endogenous stem cell differentiation *in vivo* and promote bone healing. Therefore, we synthesized matrix metalloproteinase (MMP)-degradable PEG-maleimide hydrogels functionalized with GFOGER and incorporating recombinant human BMP-2. In order to test this hypothesis, we implanted protease-degradable GFOGER-modified PEG hydrogel BMP-2 carriers within critical-sized, non-healing murine radial bone defects in order to evaluate their effects on bone regeneration.

2. Materials and methods

2.1. GFOGER-modified PEG hydrogel synthesis

GFOGER peptide, GGYGGGP$(GPP)_5$GFOGER$(GPP)_5$GPC (Activotec), four-arm, maleimide-end functionalized (>95%) PEG macromer (PEG-MAL, 20 kDa, Laysan Bio), GRGDSPC (RGD adhesive peptide), and GCRDVPMSMRGGDRCG (VPM) cross-linker peptide (AAPTEC), and rhBMP-2 (R&D Biosystems) were used. PEG-MAL hydrogels (4% wt/v) were synthesized by reacting PEG-MAL with adhesive peptides (RGD or GFOGER) followed by mixing in BMP-2 and VPM cross-linker at a volume ratio of 2:1:1:1 at the required concentrations to obtain the desired final concentrations of the adhesive peptide (0.5–2.0 mM) and BMP-2 (0.03, 0.06 or 0.3 μg per 1.5 μL hydrogel implant). The concentration of VPM used for the synthesis of each hydrogel was calculated to match the number of cysteine residues on the peptide cross-linker with the number of free (unreacted) maleimide groups remaining in the adhesive peptide-functionalized PEG-maleimide solution. The mixture of peptide-functionalized PEG-maleimide, BMP-2 and VPM cross-linker was incubated at 37 °C for 2–6 h to allow for cross-linking before adding PBS to the hydrogels. For *in vitro* studies, thin gel discs were fabricated by covering polymerizing gel solutions with sterile Sigmacote-treated coverslips. For *in vivo* studies, hydrogel (1.5 μL) was cast within 4-mm long polyimide tube sleeves with laser machined 200 μm diameter holes to improve nutrient transport and cell invasion into the defect. All hydrogels used for *in vivo* studies contained 4% (wt/v) PEG-maleimide and 2.0 mM adhesive peptide. Collagen sponges were cut with a 1 mm diameter biopsy sponge and placed within the polyimide sleeves, injected with a BMP-2 solution at an equivalent dose as was loaded in the GFOGER hydrogels and incubated for 10 min at room temperature to allow for adsorption to the collagen sponge prior to implantation.

2.2. In vitro assays

hMSCs (Lonza) were cultured in MSCGM (Lonza) and seeded (10,000 cells/cm^2) on hydrogel surfaces. Cells were cultured for up to 21 days in osteogenic media (Lonza). After 3 days of culture in osteogenic media, cells were incubated in 2 μM calcein and 4 μM ethidium homodimer for 30 min for Live/Dead staining and imaged on a Zeiss fluorescence microscope. At 14 days hMSCs were lysed and assayed for alkaline phosphatase activity (ALP) by incubating with MUP substrate. hMSCs were scraped in PBS, transferred to cold 50 mM Tris–HCl and sonicated to lyse the cells. The total protein content for each lysate sample was determined using a BCA assay kit (Thermo Scientific). Samples were diluted to the same total protein content before assaying for ALP. Samples and ALP standards were loaded into a 96-well plate, then incubated with MUP substrate at 37 °C for 1 h and read at 360 nm excitation/465 nm emission. Mineral deposition at 21 days post-induction was assayed by Alizarin Red staining and extraction. Cells were fixed in 10% formalin, rinsed in water and incubated in 2% Alizarin Red solution for 20 min. After 4 washes in water, the stained cells were scraped in 10% acetic acid and heated to 85 °C for 10 min. The supernatant was collected after centrifugation, neutralized with 10% ammonium hydroxide and read at 405 nm.

2.3. Radial defect surgery

All animal experiments were performed with the approval of the Georgia Tech Animal Care and Use Committee with veterinary supervision and within the guidelines of the Guide for the Care and Use of Laboratory Animals. B6129SF2/J wild-type male mice (8–10 week old, Jackson Laboratories) were anesthetized under isoflurane, and fur was removed from the right forelimb. The forelimb was then swabbed with chlorohexidine and alcohol and a 1.5-cm incision was made in the skin. Muscle tissue overlying the ulna and radius were blunt dissected, and 2.5 mm defects were made in the right radius using a custom-machined bone cutter, while leaving the ulna intact. Hydrogel or collagen sponge placed within polyimide sleeves were implanted into the defect by fitting the sleeve over the radius at the proximal and distal ends of the defect, so that the hydrogel or collagen sponge filled the defect space. The incision was then closed with vicryl suture and wound clips. Mice were provided with a single dose of slow-release buprenorphine for pain relief and were monitored post-surgery for signs of distress, normal eating habits and movement.

2.4. BMP-2, GFOGER in vivo retention

GFOGER and BMP-2 were labeled with Vivotag 680 and Vivotag 800 IR dyes, respectively. Labeled GFOGER and BMP-2 were incorporated into PEG hydrogels and implanted into mice as described earlier. GFOGER peptide and BMP-2 retention within the defect site ($n = 6$) was analyzed by scanning mouse forelimbs (FMT 4000) on the 680 or 790 laser channels (Perkin Elmer, 1 mm source density, 65 source points per scan). The signal was quantified by placing 3D regions of interest markers around the forelimb, using a 0.0 nM IR dye threshold and normalizing to the day 0 value.

2.5. Faxitron/μCT imaging and mechanical testing

Radial defects were imaged with the MX-20 Radiography System (Faxitron, 23 kV energy setting, 15 s scan time). For μCT scanning, a 3.2 mm length of the radius centered around the 2.5 mm radial defects was scanned in anesthetized, live subjects using a VivaCT system (Scanco Medical, 145 μA intensity, 55 kVp energy, 200 ms integration time, and 15 μm resolution). Bone formation was evaluated by contouring 2D slices to include only the radius and applying a Gaussian filter (sigma = 1, support = 1, threshold = 540 mg HA/ccm). 3D μCT reconstructions display the full 3.2 mm length of radius scanned. However, in order to ensure that only new bone formation was measured, quantification of bone volume and mineral density within the defect was performed by evaluating only the middle 2.0 mm of defect.

Torsion to failure testing was performed on 8-week radial defects ($n = 5–9$) as described [42] with modifications. The radii and ulnae were excised post-euthanasia, wrapped in PBS-soaked gauze and stored at −20 °C. On the day of testing, the bones were thawed and potted in woods–metal within potting blocks. After the ulna was cut, the potting blocks were tested using a Bose Electroforce ELF 3200 system. The radius was torqued to failure at a rate of 3° per second and the torque was measured using a 0.07 N m torque sensor (Transducer Techniques) (Fig. S3).