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Non-invasive monitoring of BMP-2 retention and bone formation in composites for bone tissue engineering using SPECT/CT and scintillation probes

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ABSTRACT

Non-invasive imaging can provide essential information for the optimization of new drug delivery-based bone regeneration strategies to repair damaged or impaired bone tissue. This study investigates the applicability of nuclear medicine and radiological techniques to monitor growth factor retention profiles and subsequent effects on bone formation. Recombinant human bone morphogenetic protein-2 (BMP-2, 6.5 μg/scaffold) was incorporated into a sustained release vehicle consisting of poly(lactic-co-glycolic acid) microspheres embedded in a poly(propylene fumarate) scaffold surrounded by a gelatin hydrogel and implanted subcutaneously and in 5-mm segmental femoral defects in 9 rats for a period of 56 days. To determine the pharmacokinetic profile, BMP-2 was radiolabeled with ^{125}I and the local retention of ^{125}I-BMP-2 was measured by single photon emission computed tomography (SPECT), scintillation probes and ex vivo scintillation analysis. Bone formation was monitored by micro-computed tomography (μCT). The scaffolds released BMP-2 in a sustained fashion over the 56-day implantation period. A good correlation between the SPECT and scintillation probe measurements was found and there were no significant differences between the non-invasive and ex-vivo counting method after 8 weeks of follow up. SPECT analysis of the total body and thyroid counts showed a limited accumulation of ^{125}I within the body. Ectopic bone formation was induced in the scaffolds and the femur defects healed completely. In vivo μCT imaging detected the first signs of bone formation at days 14 and 28 for the orthotopic and ectopic implants, respectively, and provided a detailed profile of the bone formation rate. Overall, this study clearly demonstrates the benefit of applying non-invasive techniques in drug delivery-based bone regeneration strategies by providing detailed and reliable profiles of the growth factor retention and bone formation at different implantation sites in a limited number of animals.

1. Introduction

During the past decades, the accuracy and resolution of non-invasive monitoring techniques in medicine have greatly improved due to tremendous advances in knowledge, techniques and equipment. Due to the low morbidity of these procedures, they allow repeated evaluation of normal biological processes and diseases over time with limited risks of complications. As a result of the technological advances, many of these non-invasive techniques have also become available for research in small animals [1–3].

One of the research fields that can especially benefit from non-invasive monitoring techniques is bone tissue engineering. This field strives to create living, functional bone tissue to repair large bone defects due to disease, damage, or congenital defects that would fail to heal by themselves [4]. Controlled release of growth factors involved in the natural process of bone healing has become of great importance for the local modulation of bone formation at the defect site [5]. Although many of these growth factor delivery vehicles appear promising in animal experimental settings, optimization of the vehicle properties, release profiles and site-specific pharmacological actions remains challenging.

While in vivo bone formation is relatively easy to quantify by non-invasively radiographic techniques, studying the local release kinetics of the growth factors from the delivery vehicle in vivo is more complicated. One of the moderately successful ways to monitor in vivo release profiles consists of tracing a radiolabeled protein. This technique has been applied in an invasive way by ex-vivo measuring of the radioactive protein retention after surgical removal of the implants [6–11]. Although this ex vivo method is considered the

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golden standard because of the high spatial resolution and quantitative information, it has several disadvantages. Ex-vivo counting does not allow sequential release measurements of the same implant and requires large numbers of animals [12–16]. Furthermore, simultaneous measurement of both growth factor release and bone formation is complicated, since the first event usually precedes the latter. Therefore, non-invasive nuclear medicine techniques are being explored for determining release profiles [17–22].

Combining non-invasive nuclear medicine and radiological technologies holds great potential for the optimization of growth factor delivery vehicles, since they allow sequential measurements of both growth factor release and its biological effect (i.e. bone formation). The aim of this study was to investigate the feasibility of using non-invasive techniques to simultaneously monitor both growth factor release and bone formation. Single photon emission computed tomography (SPECT) and a previously described scintillation probe setup were used to determine local retention profiles and micro-computed tomography (μCT) was used for monitoring bone induction [17,18]. Both events were monitored at an ectopic and orthotopic implantation site in rats using a local delivery vehicle containing radioiodinated recombinant human bone morphogenetic protein-2 (BMP-2).

2. Materials and methods

2.1. Experimental design

A total of 18 rats were used for the experiment according to the approved protocol by the Institutional Animal Care and Use Committee. Growth factor release profiles and bone forming capacity were studied over a period of 8 weeks in an ectopic (subcutaneous) and orthotopic (critical sized femoral defect) implantation site in 8 rats. Release profiles were determined non-invasively by SPECT/CT and scintillation probes. Bone formation was studied using μCT. The implants were removed after 8 weeks of implantation for the comparison of non-invasive and ex-vivo measurements. Additional femoral defects without an implant were used as controls for the autologous bone formation. The bone formation in these controls was only determined using the ex-vivo μCT.

2.2. BMP-2 radioiodination

Recombinant human BMP-2 (purchased as part of an Infuse® research kit, Medtronic Sofamor Danek, Minneapolis, MN) dissolved in a BMP-2 buffer (5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, 0.01% Tween80 and pH 4.5) was radiolabeled with I^{125} using Iodo-Gen® precoated test tubes according to the manufacturer's instructions (Pierce, Rockford, IL). Briefly, 100 μl of a 1.43 mg/ml BMP-2 solution, 20 μl of a 0.1 M NaOH solution and 2 mCi NaI^{125} were added to an Iodo-Gen® coated test tube and incubated for 30 minutes at room temperature. The radiolabeled protein was then separated from free ^{125}I by 24 h dialysis (10 kDa molecular weight cutoff (MWCO) Slide-A-Lyzer®, Pierce) against the BMP-2 buffer and subsequently concentrated in a Vivaspin device (10 kDa MWCO, Sartorius AG, Germany). The final ^{125}I-BMP-2 solution had an estimated concentration of 1.5 μg/μl, an activity of 4.0 μCi/μg and a trichloroacetic acid (TCA) precipitability of 99.8% precipitable counts.

2.3. BMP-2 delivery vehicle

The sustained delivery vehicle consisted of BMP-2-containing poly (lactic-co-glycolic acid) (PLGA) microspheres embedded into a poly (propylene fumarate) (PPF) rod which was surrounded by a cylindrical gelatin hydrogel. The materials and scaffold design were based on previous work on BMP-2 binding and extended protein release [23]. The PLGA (acid end-capped, 50:50 L:G ratio, Mw 23 kDa, Medisorb®, Lakeshore Biomaterials, AL) microspheres were fabricated using a double-emulsion-solvent-extraction technique as previously described [23,24]. The starting amount for the microsphere fabrication was 7.4 mg BMP-2/g PLGA with a 1:4 hot:cold ratio of ^{125}I-BMP-2/BMP-2. Based on the ^{125}I counts before and after the fabrication procedure, the microsphere entrapment efficiency was estimated at 85% or 1.1 μg BMP-2 per mg PLGA.

The microsphere/PPF composites were fabricated by photocrosslinking PPF with a Mw of 5800 Da and a polydispersity (PI) of 2.0 with N-vinylpyrrolidinone (NVP, Acros, Pittsburgh, PA) using the photoinitiator bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO, Ciba Specialty Chemicals, Tarrytown, NY) as previously described [23,25]. This resulted in cylindrical microsphere/PPF rods with a diameter of 1.6 mm, a length of 6 mm, an average activity of 5.3 ± 0.3 μCi and an estimated BMP-2 loading of 6.5 ± 0.4 μg. The rods were sterilized by ethanol evaporation and frozen down at −20 °C until use.

The microsphere/PPF composites were combined with a cylindrical shell composed of a gelatin (type A, 300 bloom, derived from acid-cured tissue, Sigma-Aldrich) hydrogel just before implantation. The gelatin hydrogels had an outer diameter of 3.5 mm and an inner diameter of 1.6 mm and were sterilized in 70% alcohol. Prior to implantation, the BMP-2 loaded microsphere/PPF composite were inserted into the gelatin cylinders.

2.4. Surgical procedure

A total of nine male 12-week-old Sprague Dawley rats (weight 323 ± 9 g) were obtained from a professional stockbreeder (Harlan Sprague Dawley, Inc., Indianapolis, In) at least 1 week prior to the start of the experiment. Anesthesia was provided by an intramuscular injection of a ketamine/xylazine mixture (45/10 mg/kg) and the surgical sites were shaved and disinfected. For the orthotopic implant, a 2 cm skin incision was made along the lateral site of the right limb. By blunt dissection between the biceps femoris and the vastus lateralis muscle, the femur was exposed and circumferentially freed from muscles. A pre-drilled polyethylene plate ($l \times h \times w$, 22 × 3 × 4 mm) was fixed to the femur with 1 mm Kirchner-wires (Zimmer, Warsaw, IN). Subsequently, a 5-mm segmental defect was created using a surgical drill, which was filled with the microsphere/PPF/gelatin implant. No additional fixation was required to keep the slightly oversized implant (6 mm) with a PPF/microsphere rod diameter equal to the intra-medullary canal (1.6 mm) in place in the 5 mm femoral defect between inner 2 K-wires (7 mm apart). In the controls, the femoral defects were left empty. In addition to the orthotopic implant, the rats receive two ectopic implants in subcutaneous pockets in the lower left limb and the thoracolumbar area in the back. The limb pocket was filled with a ^{125}I-BMP-2 implant and the pocket in the back with a non-radioactive implant.

2.5. SPECT/CT imaging and image analysis

Animal imaging was performed with a SPECT/CT system (X-SPECT, Gamma Medica-Ideas, Inc., Northridge, CA, USA) equipped with a single X-ray tube-detector unit and two identical high-resolution gamma cameras. The gamma cameras were collimated by low-energy, high-resolution, parallel-hole collimators with a 12.5 cm × 12.5 cm field of view. Fixed system settings and imaging geometries were used throughout the course of the study. The maximum resolution of the SPECT and CT system are 1–2 mm and 50 μm, respectively. The counting rate linearity of the SPECT system was determined by scanning microcentrifuge tubes containing 20 μl of ^{125}I-BMP-2 solutions with activities varying from 0.041 to 12 μCi. The activity of the tube and the number of acquired counts showed a linear correlation with an R^2 of 1.000.

The animals were imaged under ketamine/xylazine sedation (20/5 mg/kg) postoperatively and at days 3, 7, 14, 21, 28, 35, 42, 49 and 56. During imaging, the rats remained stationary on a platform in the center of the system, while the X-ray unit and gamma cameras rotated