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Non-invasive tri-modal visualisation *via* PET/SPECT/μCT of recombinant human bone morphogenetic protein-2 retention and associated bone regeneration: A proof of concept

Gry Hulsart-Billström[a,⁎,1], Ram Kumar Selvaraju[b,1], Sergio Estrada[b], Mark Lubberink[c], Veronika Asplund[b], Kristoffer Bergman[d], Richard Marsell[a], Sune Larsson[a], Gunnar Antoni[e]

[a] Department of Orthopedics, Uppsala University, Uppsala, Sweden
[b] Department of Medicinal Chemistry, Preclinical PET-MRI Platform, Uppsala University, Uppsala, Sweden
[c] Department of Nuclear Medicine & PET, Uppsala University, Uppsala, Sweden
[d] TERMIRA, Stockholm, Sweden
[e] Department of Medicinal Chemistry, Uppsala University, Uppsala, Sweden

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A B S T R A C T

Bone morphogenetic proteins (BMP's) are vital for bone and cartilage formation, where bone morphogenetic protein-2 (BMP-2) is acknowledged as a growth factor in osteoblast differentiation. However, uncontrolled delivery may result in adverse clinical effects. In this study we investigated the possibility for longitudinal and non-invasive monitoring of implanted [^{125}I]BMP-2 retention and its relation to ossification at the site of implantation. A unilateral critically sized femoral defect was produced in the left limb of rats while the right femur was retained intact as a paired reference control. The defect was filled with a hyaluronan hydrogel with 25% hydroxyapatite alone (carrier control; $n = 2$) or combined with a mixture of [^{125}I]BMP-2 (150 μg/ml; $n = 4$). Bone formation was monitored using micro computed tomography (μCT) scans at 1, 3, 5, 7, 9 and 12 weeks. The retention of [^{125}I]BMP-2 was assessed with single photon emission computed tomography (SPECT), and the bone healing process was followed with sodium fluoride (Na^{18}F) using positron emission tomography (PET) at day 3 and at week 2, 4, and 6. A rapid burst release of [^{125}I]BMP-2 was detected *via* SPECT. This was followed by a progressive increase in uptake levels of [^{18}F]fluoride depicted by PET imaging that was confirmed as bone formation *via* μCT. We propose that this functional, non-invasive imaging method allows tri-modal visualisation of the release of BMP-2 and the following *in vivo* response. We suggest that the potential of this novel technique could be considered for preclinical evaluation of novel smart materials on bone regeneration.

1. Introduction

The growing body of knowledge regarding the mechanisms involved in bone regeneration is enabling the development of a plethora of new biomaterials tailored to promote safe and controlled bone formation in clinical settings. This new generation of smart materials is designed to induce bone regeneration solely by its properties at lowest possible concentration of growth factors with minimum side effects [1–5]. Thus, there is a need for a better understanding of how the *in situ* activity of a smart material correlate with an *in vivo* response. In this context, a non-invasive methodology for visualisation and quantification of the bone healing process would be of great importance.

One such example of a non-invasive technique currently used for the clinical investigation of functional bone imaging is positron emission tomography (PET), along with computed tomography (CT) for anatomical information [6]. PET and sodium [^{18}F]fluoride gives high-resolution PET images and reliable quantification of tracer uptake in bone as a measure of osteoblast activity. [^{18}F]fluoride has the ability to substitute the inorganic components of the bone matrix, namely hydroxide and phosphate, which are found at elevated concentrations at the site of active mineralisation [7,8]. Clinically, [^{18}F]fluoride and PET-CT is often used in the investigation of malignancies in bone tissue [9].

Single-photon emission computed tomography (SPECT) is another clinical modality for bone imaging. SPECT, in contrast to PET, produces a qualitative measure of osteoblastic activity. Ventura et al. compared both techniques for monitoring of bone regeneration by using [^{99m}Tc]

⁎ Corresponding author at: Department of Surgical Sciences, Orthopedics, Uppsala University, Akademiska Sjukhuset Ingång 70 3tr, 751 85 Uppsala, Sweden.
E-mail address: gry.hulsart@surgsci.uu.se (G. Hulsart-Billström).

[1] G. Hulsart-Billström and R. K. Selvaraju contributed equally to this work and should be regarded as joint first authors.

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hydroxymethylenediphosphate in SPECT, and [^{18}F]fluoride in PET. They concluded that PET had a higher sensitivity than SPECT when used in the detection of bone formation [10]. In addition, Ventura et al. explored the use of PET as a method to monitor the *in vivo* response to a calcium phosphate cement that were releasing BMP-2 in a calvarial defect model. They found that there was a positive correlation between the uptake of [^{18}F] fluoride and the volume of *de novo* bone [11].

Computed tomography has been a tool for bone imaging for almost half a century and has since its development by Hounsfield et al. [12] experienced great advances with nano-size resolution imaging and temporal *in vivo* scanning. Bone has been the most common subject of CT scans due to its high density and the field of bone morphometric and bone development is extensively developed and standardised.

Bone regeneration is a complex process that is tightly regulated through the orchestrated release of cytokines. BMP-2 is one of the cytokines produced and released during this process [13,14]. It has strong osteo-inductive effects, which drive the migration of osteoprogenitor cells to the site of bone regeneration, and their subsequent differentiation. These properties demonstrate its high potential as a growth factor, and therefore it is thoroughly investigated as a potential growth factor in combination with scaffolds for regenerative strategies [5,15].

In this proof of concept study, a commercially (TERMIRA) available hydrogel composed of hyaluronan (HA) was used to facilitate bone regeneration. We employed a novel combination of *in vivo* imaging modalities for evaluation of biomaterials, cytokines administered and the *in situ* response to enhance bone regeneration. A critically sized segmental rat femoral defect was used as a temporal *in vivo* model. We fused the methods of Kempen, Ventura, Lienemann and van de Watering [11,16–19] and advanced the technique to apply a tri-modal dual-isotope approach coupling SPECT and PET with traditional μCT validation.

We hypothesise that the combination of PET/SPECT/μCT could be considered for preclinical evaluation of novel smart materials on bone regeneration providing a detailed non-invasive and longitudinal monitoring of bone healing.

2. Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Aldrich, Stockholm, Sweden.

2.1. Radiolabeling of BMP-2

The radiolabeling was done according to the Hunter and Greenwood method using iodine-125 (I^2) that has a physical half-life of 59.4 days and decays 100% by electron capture [20]. In short, 10 μl of 1,5 mg/ml BMP-2 (InductOs, Medtronic BioPharma, Netherlands) was added to a low binding protein eppendorf tube (VWR, Stockholm, Sweden) followed by 18 μl of (I^2) iodine-125 with an radioactivity of 66,7 MBq 20 μl of Chloramine T (1 mg/ml) (Sigma, No·S-8890, St. Louis, MO, USA) was added to start the reaction. The solution was vortexed for 2 min and 20 μl of sodium metabisulfite (Sigma, No. S-8890, St. Louis, MO, USA) was added to stop the reaction, resuming the vortex mixing for two additional minutes. Phosphate-buffered saline (PBS) was added to a final volume of 500 μl and the solution was transferred to a NAP-5 column (GE Healthcare, Uppsala, Sweden) to separate the [^{125}I]BMP-2 from the free (I^2) [^{125}I]iodine. The column was pre-coated with 1% bovine serum albumin (BSA) in PBS, to minimise the [^{125}I]BMP-2 unspecific binding. To collect the [^{125}I]BMP-2 from the column five repeats of 500 μl of PBS were added to the columns to produce five fractions. The high molecular fraction with the highest activity was used for the study. The [^{125}I]BMP-2 was stored at 4 °C until the preparation of the materials the next day.

2.2. Hydrogel preparation

A commercial hyaluronan aldehyde-poylyvinyl alcohol (HAA/PVAH) system previously described by Bergman et al. was kindly provided by TERMIRA (Auxigel; Termira, Stockholm, Sweden) [9]. In short, the HAA component and PVAH component were dissolved in PBS (pH 7.4) at final concentration 15 mg/ml for HAA and 4.3 mg/ml for PVAH. The HAA were sterilised through a 0.45 μm filter due to its viscous characteristics and PVAH was sterilised through a 0.22 μm filter. The amount of 25% *w/v* of hydroxyapatite powder (Captal, Plasma Biotal, Buxton, UK) with an average particle size of 3.39 μm and Ca/P ratio of 1.67 was sterilised by incubation at 200 °C for 2 h. A 1.5 μg/μl BMP-2 (InductOs, Medtronic, BioPharma, Netherlands) stock solution in formulation buffer (2.5% glycine, 0.5% sucrose, 0.01% polysorbate 80, 5 mM sodium chloride and 5 mM L-glutamic acid, pH 4.5) was prepared according to the manufacturer's instructions. A 2:3 ratio of [^{125}I]BMP-2 (0.03 μg/μl) and BMP-2 (1.5 μg/μl) was mixed, resulting in approximately 2% of the BMP-2 being radiolabeled. The sterile material was used to prepare the HA aldehyde component (15 mg/ml of HAA and 250 mg/ml HAP) and the PVA hydrazide component (2.5 mg/ml PVAH, 250 mg/ml HAP and 0.375 mg/ml BMP-2). The components were loaded into 1 ml syringes at 3:2 volume ratios of HAA and PVAH. The syringes were connected with a sterile adapter and the components were mixed at room temperature (20 °C) back and forth 30 times for 15 s, as previously described [21,22]. As a result, 300 μl of chemically cross-linked hydrogel premix was obtained containing 1% *w/v* polymer, 25% w/v HAP and 0.15 mg/ml [^{125}I]BMP-2, with a radioactivity of approximately 800 KBq.

2.3. Animal model and surgical procedure

The animal study was approved by the Uppsala Committee of Animal Research Ethics (C76/13), according to the Federation of European Laboratory Animal Science Association's guidelines. The hydrogel was injected into a critically sized segmental defect in the (left femur) followed by stabilisation with an external fixator, while the intact right femur was used as paired control for each animal [23]. Male Sprague Dawley (SPRD) rats (400–450 g) were randomised into two groups. The defects were either filled with a control hydrogel carrier ($n = 2$) as it has previously shown no bone formation without addition of growth factors [24,25], or with the hydrogel combined with 30 μg of [^{125}I]BMP-2 ($n = 4$; 80 kBq) at a concentration of 150 μg/ml (~75 μg/kg), which has proven earlier to induce extensive bone formation.

The animals were anesthetised on a facemask with 0.3 l/min oxygen, 1–2.5% isoflurane, and 0.8 l/min nitrous oxide and placed on a 37 °C heat pad during surgery (Isoba vet, Schering-Plough, USA). The left femur was shaved and sterilised with Chlorhexidine Ethanol (5 mg/ml). One dosage of 225 mg/kg antibiotics (Zinacef, GlaxoSmithKline AB, Sweden) was given subcutaneously. The femur was exposed by making a longitudinal lateral skin incision followed by blunt dissection in-between m. vastuslateralis and m. biceps femoris. Four 0.75 mm bicortical holes were drilled (Dremel multi, Robert Bosch Tool Corporation, Germany) by placing a guide on the lateral side of the femur that was centred mid-diaphyseal using a purpose-built forceps. Each drill hole was tapped followed by insertion of 1 mm stainless steel pins. The pins were pierced through the skin and locked into an aluminium and stainless steel external fixator. An oscillating saw (Stryker total performance system, TPS sagittal saw with 5 mm saw blade) was used to create a 5 mm mid-diaphyseal defect guided by a saw guide to standardise the defect. Saline was administered to prevent tissue necrosis during the sawing. The resected bone was extracted with a clamp and debris was washed out of the defect with saline. Two sutures (Polysorb, Tyco Healthcare, Gosport, UK) were applied in the fascia layer, with the proximal left untied. The volume of 200 μl of hydrogel with a radioactivity of approximately 600 KBq and a dose of 30 μg of BMP-2 was injected into the defect, after which the second fascia (4-0

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