Acta Biomaterialia 10 (2014) 2250–2258

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates

Ciara M. Murphy [a,b,*], Aaron Schindeler [a,b], John P. Gleeson [c], Nicole Y.C. Yu [a,b], Laurence C. Cantrill [a,b], Kathy Mikulec [a], Lauren Peacock [a], Fergal J. O'Brien [c,d,e], David G. Little [a,b]

[a] Orthopaedic Research & Biotechnology Unit, The Children's Hospital at Westmead, Sydney, NSW, Australia
[b] Discipline of Paediatrics and Child Health, Faculty of Medicine, University of Sydney, Sydney, NSW, Australia
[c] Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland
[d] Trinity Centre for Bioengineering, Trinity College, Dublin, Ireland
[e] Advanced Materials and BioEngineering Research Centre (AMBER), RCSI & TCD, Dublin, Ireland

ARTICLE INFO

Article history:
Received 3 October 2013
Received in revised form 16 December 2013
Accepted 14 January 2014
Available online 21 January 2014

Keywords:
Zoledronic acid
Bisphosphonate
rhBMP-2
Collagen
Hydroxyapatite

ABSTRACT

An emerging paradigm in orthopedics is that a bone-healing outcome is the product of the anabolic (bone-forming) and catabolic (bone-resorbing) outcomes. Recently, surgical and tissue engineering strategies have emerged that combine recombinant human bone morphogenetic proteins (rhBMPs) and bisphosphonates (BPs) in order to maximize anabolism and minimize catabolism. Collagen-based scaffolds that are the current surgical standard can bind rhBMPs, but not BPs. We hypothesized that a biomimetic collagen–hydroxyapatite (CHA) scaffold would bind both agents and produce superior in vivo outcomes. Consistent with this concept, in vitro elution studies utilizing rhBMP-2 ELISA assays and scintillation counting of ^{14}C-radiolabeled zoledronic acid (ZA) confirmed delayed release of both agents from the CHA scaffold. Next, scaffolds were tested for their capacity to form ectopic bone after surgical implantation into the rat hind limb. Using CHA, a significant 6-fold increase in bone volume was seen in rhBMP-2/ZA groups compared to rhBMP-2 alone, confirming the ability of ZA to enhance rhBMP-2 bone formation. CHA scaffolds were found to be capable of generating mineralized tissue in the absence of rhBMP-2. This study has implications for future clinical treatments of critical bone defects. It demonstrates the relative advantages of co-delivering anabolic and anti-catabolic agents using a multicomponent scaffold system.

1. Introduction

Bone and fracture repair occurs naturally through a series of anabolic and catabolic processes [1]. However, in the cases of severe trauma or disease, surgical intervention is required. Bone grafts and bone graft substitutes are used in the surgical repair and reconstruction of critical defects. Currently the clinical "gold standard" approach is the use of autografts. This approach is not without complications, such as donor site morbidity, quantity of available tissue and the need for a second surgery. The use of allografts can overcome these obstacles. However, there is a worldwide shortage of donors which, together with an associated risk of disease transmission, hinders allografts as an ideal treatment option [2].

Bone tissue engineering aims to develop bone graft replacements that can repair bone defects without a need for allografts or autografts. Currently, collagen matrices loaded with anabolic agents are clinically utilized as bone graft substitutes. Recombinant human bone morphogenetic proteins (rhBMPs) are potent anabolic agents that are in clinical use for the treatment of non-union and critical-sized bone defects. Treatment with rhBMPs facilitates the differentiation of mesenchymal progenitors into bone-forming osteoblasts [3]. This leads to robust induction of new bone formation. However, in non-load-bearing defects or when used at high local doses, rhBMPs have been shown to both directly and indirectly induce osteoclast differentiation [4,5]. This is problematic as it can lead to the premature resorption of bone [5–7].

An emerging paradigm for orthopedics is that bone formed from an anabolic stimulus can be maximized by use of an anti-catabolic agent [8–11]. Bisphosphonates (BPs) are anti-catabolics clinically used in the treatment of osteoporosis and metabolic bone diseases, and have also been shown to minimize bone loss due to unloading or stress shielding [12]. BPs have a high affinity for the hydroxyapatite

* Corresponding author at: Orthopaedic Research & Biotechnology Unit, The Children's Hospital at Westmead, Sydney, NSW, Australia. Tel.: +61 2 98451451; fax: +61 2 98453078.
E-mail address: ciara.murphy@sydney.edu.au (C.M. Murphy).

http://dx.doi.org/10.1016/j.actbio.2014.01.016
1742-7061/

(HA) within bone mineral. Once bound, they potently inhibit osteoclast-mediated resorption [13,14]. Only the osteoclasts are able to mobilize and internalize BPs bound with high affinity to the bone mineral. As the osteoclasts resorb the bone, they internalize the BPs, leading to inhibition of osteoclast activity and/or osteoclast apoptosis [15–18].

To date, studies utilizing combined anabolic and anti-catabolic treatments have typically relied on systemic BP delivery [12,19]. However, there are a number of adverse affects associated with systemic dosing of PBs, including stomach ulceration (oral BPs) and flu-like symptoms (intravenous dosing) [20,21]. In rare cases major complications have been reported, such as renal failure and osteonecrosis of the jaw, although these are typically linked to underlying kidney diseases or dental problems [22]. Local delivery has emerged as an appealing alternative to systemic delivery as it avoids these complications and can lower the absolute BP dose required. Furthermore, it simplifies treatment for the patient as both drug delivery and surgery are combined into one procedure rather than requiring post-surgical systemic/oral administration. Multiple studies have utilized polymeric-based (poly-L-lactide, PLLA; poly(lactic-*co*-glycolic) acid, PLGA) carriers to demonstrate synergistic benefits for co-delivering rhBMPs and BPs locally [23,24]. However, these materials can be associated with problems, including the release of acidic degradation by-products that can alter the pH in surrounding tissue. In turn, this can cause adverse tissue and inflammatory reactions [25,26].

Collagen, as a naturally derived biomaterial, has many advantages over most synthetically derived polymers. It shows superior biocompatibility, biodegradability, interconnected porous architecture [16] and a capacity to bind rhBMPs. As such, it has been adopted as an rhBMP delivery system now in clinical use (rhBMP-2: Medtronic INFUSE®; and rhBMP-7: Olympus OP-1® bone graft). However, while collagen provides structural stability to a number of endogenous tissues, including bone, scaffolds comprising collagen possess poor load-bearing capabilities.

This study trialed a recently developed composite collagen–hydroxyapatite (CHA) scaffold that incorporates both collagen and HA in a porous scaffold matrix [27]. These materials are the two major constituents of bone and a logical choice as the basis of a biomimetic scaffold capable of supporting and promoting bone regeneration [28]. Furthermore, the CHA scaffold combines the advantages conferred by the mechanical strength of ceramics with the biological advantages of collagen. The CHA scaffold has been developed using a patented fabrication process [20] and incorporates features used in the development of a range of collagen-based scaffolds optimized in terms of composition [26,29], cross-linking density [30,31] and pore architecture [32–35], for use in bone tissue engineering applications. The CHA scaffold has 99% porosity with higher pore interconnectivity than standard collagen, measured in terms of scaffold permeability (0.4×10^{-9} $m^4 N^{-1} s^{-1}$ vs. 4.5×10^{-9} $m^4 N^{-1} s^{-1}$). The addition of HA confers improved mechanical strength over collagen alone (1.3 kPa vs. 0.5 kPa). This CHA scaffold has previously facilitated healing of a critical-sized rat calvarial defect without the addition of exogenous pro-osteogenic factors [36].

It was hypothesized that this biphasic collagen/HA scaffold would show superior binding of an rhBMP-2/BP combination. Collagen facilitates rhBMP-2 binding [37], and BPs have a high affinity for HA [38]. It was further speculated that this combination of collagen, HA, rhBMP-2 and the BP zoledronic acid (ZA) would achieve superior bone formation. The first aim of this study was to investigate the binding and retention of rhBMP-2 and ZA to the biphasic components of the scaffold in comparison to control collagen scaffolds. The second aim was to then test the efficacy of this system in an ectopic bone formation model in a rodent hind limb.

2. Materials and methods

2.1. Pharmaceuticals

rhBMP-2 from the INFUSE® Bone Graft Small Kit was purchased from Medtronic (Memphis, TN, USA). ZA was purchased from AXXORA, LLC (San Diego, CA, USA). For in vitro studies, a stock solution of ^{14}C-radiolabeled ZA (^{14}C-ZA) with a specific activity of 7.027 MBq mg^{-1} was supplied by Novartis Pharma AG (Switzerland). This was diluted with unlabeled ZA to generate a 1 mg ml^{-1} solution with a specific activity of 703 kBq mg^{-1}. AlexaFluor 647 was purchased from Life Technologies (Victoria, Australia).

2.2. Scaffold fabrication

The CHA scaffold was fabricated as previously described [36]. In brief, collagen slurries were produced by the homogenization of fibrillar collagen (Collagen Matrix, Franklin Lakes, NJ, USA) within a 0.5 M acetic acid solution. Slurries were homogenized in a reaction vessel and cooled to 4 °C by a WK1250 cooling system (Lauda, Westbury, NY, USA) using an overhead blender (IKA Works Inc., Wilmington, NC, USA). In parallel, HA particles with a mean diameter of 5 μm (Plasma Biotal Limited, North Derbyshire, UK) were suspended in a 0.5 M acetic acid solution. Collagen concentration was 0.1 g ml^{-1} and HA concentration was 0.2 g ml^{-1}. The final composite slurry was produced by lyophilization after being pipetted into a stainless steel pan (125 × 125 mm, grade 304 SS) and cooled to −40 °C at a constant cooling rate of 0.9 °C min^{-1}. After freezing, ice crystals were removed by sublimation for 17 h at 0 °C and 200 mtorr [32,39]. This produced scaffolds with a pore size of ~100 μm. Dehydrothermal (DHT) cross-linking treatment was carried out as previously described [40] under a vacuum of 0.05 bar at a temperature of 120 °C for 24 h. CHA scaffolds were sliced into 6 mm × 3 mm sections for both in vitro and in vivo analysis.

A porous collagen sponge was used as a control for in vitro investigations. Rectangular sponges (6 mm × 3 mm) were sliced from 4 mm thick collagen sheets supplied from INFUSE® Bone Graft Small Kit (Medtronic, USA). Each scaffold was loaded with 10 μg rof hBMP-2. Following the manufacturer's instructions, rhBMP-2 solution (20 μl × 1 mg ml^{-1}) was dripped onto the collagen scaffold 20 min prior to surgical insertion.

2.3. BP binding to CHA and collagen scaffolds

Fluorescently labeled BP (Pamidronate, PAM) was used to assess the binding of the BP within the CHA and collagen. PAM was fluorescently labeled with a commercially available AlexaFluor 647 (Life Technologies, Victoria, Australia) according to the manufacturer's protocol. The resultant fluorescently labeled BP was termed AlexaPam 647. PAM has a free amine group and can be readily labeled using protein labeling kits, as opposed to the nitrogen-containing ring structure of the ZA side-chain. The addition of a die moiety does not compromise the "bone hook" involved with mineral avidity, allowing it to be used to investigate BP binding interactions in vitro.

CHA scaffolds and collagen sponges were loaded with 2 μg of AlexaPam 647 and washed for 24 h in phosphate-buffered saline (PBS) at 37 °C. The scaffolds were imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems, NSW, Australia), both pre- and post-washing.

2.4. BP elution from CHA and collagen scaffolds

Release of BP from both CHA and collagen scaffolds was measured using radiolabeled ZA (^{14}C-ZA). Specimens loaded with