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Nanoparticulate mineralized collagen scaffolds induce *in vivo* bone regeneration independent of progenitor cell loading or exogenous growth factor stimulation

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ABSTRACT

Current strategies for skeletal regeneration often require co-delivery of scaffold technologies, growth factors, and cellular material. However, isolation and expansion of stem cells can be time consuming, costly, and requires an additional procedure for harvest. Further, the introduction of supraphysiologic doses of growth factors may result in untoward clinical side effects, warranting pursuit of alternative methods for stimulating osteogenesis. In this work, we describe a nanoparticulate mineralized collagen glycosaminoglycan scaffold that induces healing of critical-sized rabbit cranial defects without addition of expanded stem cells or exogenous growth factors. We demonstrate that the mechanism of osteogenic induction corresponds to an increase in canonical BMP receptor signalling secondary to autogenous production of BMP-2 and -9 early and BMP-4 later during differentiation. Thus, nanoparticulate mineralized collagen glycosaminoglycan scaffolds may provide a novel growth factor-free and *ex vivo* progenitor cell culture-free implantable method for bone regeneration.

1. Introduction

Skeletal regenerative medicine emerged as a field of investigation to address the clinical need for reconstruction of osseous deficiencies secondary to congenital, traumatic, and post-oncologic conditions. Although reconstructive surgical techniques have undergone significant advances, correction still depends on the availability of tissue within the patient and the morbidity that a patient can sustain from tissue transfer [1,2]. Non-autologous replacement in the form of allograft and alloplast are fraught with problems including resorption, exposure, and infection [3–5]. These limitations demonstrate a significant need for establishing alternative methods for bone replacement.

Approaches to bone tissue engineering incorporate three elements: osteogenic cells, growth factors, and scaffolding material [6,7]. Cell types are usually adult tissue-derived stem cells and osteogenic growth factors are frequently members of the bone morphogenetic protein (BMP) family [8]. Mechanistically, BMP dimers promote osteogenic differentiation by binding to BMP receptor (BMPR) complexes and activating intracellular signaling cascades in osteoprogenitor cells [9]. Depending on the method of BMPR oligomerization, activation of the canonical or non-canonical pathways may occur. In the canonical pathway, the receptor Smads (Smad 1/5/8) are recruited and phosphorylated. Phosphorylated receptor Smads associate with co-Smad (Smad 4) and translocate to the nucleus to activate transcription. In the non-canonical pathway, ERK, p38 MAPK, and PI3K/Akt are activated. Regulation of each BMP receptor signaling may occur at the ligand level, receptor level, or via intracellular molecules [10–12]. Secreted homodimeric BMP

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antagonists such as noggin, chordin, and the DAN/Cerberus family of proteins sequester BMP ligands [13,14]. The pseudoreceptor BMP and activin bound protein (BAMBI) inhibits the effects of the activated receptors and is expressed in osteoblasts [15]. Intracellular negative or positive regulation can also occur within or between the canonical and noncanonical pathways with expression of inhibitory Smad proteins.

Clinically, the Food and Drug Administration (FDA) has approved two BMPs for usage in bone defects: BMP-2 and -7 [16–18]. However, complications such as soft tissue swelling, ectopic bone formation, resorption of adjacent bone, and long term effects on maxillary growth have all been reported [19–21]. Such drawbacks highlight the need for alternative methods for augmenting osteogenesis without reliance on supraphysiologic dosages of growth factors.

Scaffolding material, once considered to be inert or passive supporters of biological processes, are increasingly designed to have instructive properties that promote osteogenesis depending on the material, porosity, and ability to mimic the native extracellular matrix (ECM) [6]. Although a multitude of scaffolds have been reported in the literature comprised of ceramic, biodegradable polymers, extracellular matrix components, or combinations thereof, the quest for the ideal scaffold is yet to be complete. Classic limitations include production of inflammatory acid metabolites by biodegradable polymers and structural contraction of collagen scaffolds [22–24]. Although ceramic composites, such as β-tricalcium phosphate (β-TCP) and hydroxyapatite, are both osteoconductive and osteoinductive, their utility is limited by variable resorption rates or brittle mechanical properties [25]. We recently reported that combining both the organic and inorganic components of the ECM in the form of a novel nanoparticulate mineralized collagen glycosaminoglycan (MC-GAG) scaffold results in a highly osteogenic and structurally stable scaffold that stimulates osteogenic differentiation of human mesenchymal stem cells (hMSCs) by autogenously stimulating the canonical BMPR signaling pathway [26,27]. In this work, we evaluate the therapeutic potential of MC-GAG in bone regeneration by investigating *in vivo* calvarial bone healing in a rabbit cranial defect model.

2. Materials and methods

2.1. Fabrication of non-mineralized and mineralized collagen scaffolds

Col-GAG and MC-GAG scaffolds were prepared using the lyophilization process described previously [28]. Briefly, a suspension of collagen and GAGs were produced by combining microfibrillar, type I collagen (Collagen Matrix, Oakland, NJ) and chondroitin-6-sulfate (Sigma–Aldrich, St. Louis, MO) with and without calcium salts (calcium nitrate hydrate: $Ca(NO_3)_2{\bullet}4H_2O$; calcium hydroxide: $Ca(OH)_2$, Sigma–Aldrich, St. Louis, MO) in a solution of acetic acid (Col-GAG) or phosphoric acid (MC-GAG). The suspension was frozen using a constant cooling rate technique (1 °C/min) from room temperature to a final freezing temperature of −10 °C using a freeze dryer (Genesis, VirTis) and sublimated under vacuum (<200 mTorr, 0 °C). Scaffolds were crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Sigma Aldrich) and N-hydroxysuccinimide (NHS, Sigma Aldrich) in distilled, deionized water for 2 h at room temperature and washed with PBS [29]. The height of the scaffold specimens used in this study were 5.8 ± 0.4 mm. Scaffold porosity was 85 ± 3% [30], pore size was 156 ± 6 μm [30,31], and morphology consisted of isotropic pores with a transverse:longitudinal pore aspect ratio of 0.95 ± 0.01 [31] as we previously reported. Disks 8 mm in diameter were prepared for *in vitro* studies using punch biopsies. Disks 14 mm in diameter were prepared for *in vivo* studies.

2.2. Animals and cell culture

2.2.1. Isolation of rabbit bone marrow stromal cells and cell culture for in vitro studies

New Zealand White rabbits (2–3 months old, cared for in compliance with the USDA Animal Welfare Act and PHS Policy for the Humane Care and Use of Laboratory Animals) were injected with 3000 U heparin and 390 mg of pentobarbital solution (Fatal-Plus®, 390 mg/ml). Bone marrow was obtained from long bones via aspiration and resuspended in PBS by successive passages through 18 and 20 gage syringe needles. Cells were cultured in α-MEM (Cellgro Mediatech, Manassas, VA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 2 mM L-glutamine (Fisher Scientific, Waltham, MA), and 100 IU/mL penicillin/100 μg/mL streptomycin (Fisher Scientific) at 37 °C for 2 days. Adherent cells were identified as stromal cells. At passage 3, 3×10^5 BMSCs were seeded onto 8-mm Col-GAG and MC-GAG scaffolds in proliferation media. 24 h after seeding, proliferation media was exchanged for osteogenic differentiation media consisting of 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 0.1 μM dexamethasone. Scaffolds were untreated or treated with rhBMP-2 at a concentration of 50 ng/mL. Media and BMP-2 were changed every 2–3 days during culture.

2.2.2. In vivo rabbit cranial defect reconstruction

Approximately three weeks prior to creation of the parietal defect, 26 female New Zealand White rabbits (2–3 months old) underwent bone marrow harvest from the ilium as described above. Preoperatively, rabbits were injected subcutaneously with enrofloxacin (5 mg/kg) and acepromazine (1 mg/kg). Anesthesia was maintained with isoflurane gas (1.5–3%) during the procedure and pain control was performed with buprenorphine 0.05 mg/kg and carprofen 4 mg/kg subcutaneous injection. Isolated BMSCs were cultured as described above and 2×10^6 cells were seeded onto 14-mm Col-GAG and MC-GAG scaffolds in proliferation media. 24 h after seeding, proliferation media was exchanged for osteogenic differentiation media consisting of 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 0.1 μM dexamethasone. Scaffolds were untreated or treated with rhBMP-2 at a concentration of 50 ng/mL for one week. Media and BMP-2 were changed every 2–3 days.

The rabbits were divided into seven groups (3–4 rabbits per group): 1) defect without reconstruction, 2) Col-GAG scaffold only, 3) Col-GAG seeded with BMSCs *ex vivo*, 4) Col-GAG seeded with BMSCs and rhBMP-2 *ex vivo*, 5) MC-GAG scaffold only, 6) MC-GAG seeded with BMSCs *ex vivo*, 7) MC-GAG seeded with BMSCs and rhBMP-2 *ex vivo*. The head of each rabbit was shaved and disinfected with Betadine. The cranial surface was exposed by a midline incision and the overlying parietal periosteum was dissected off of the calvarium. For each rabbit, a 14 mm full thickness, extradural defect was created by a hand powered trephine and the bone was lifted away without injury to the dura [32]. One scaffolds was implanted for each rabbit and the incision was closed with 4-0 nylon sutures.

Twelve weeks after implantation, the rabbits were euthanized by intravenous injection of 1 mL of pentobarbital solution (Fatal-Plus®, 390 mg/ml) intravenously via the marginal ear vein. The previous incision was then reopened and the calvarium was exposed. The calvarium including the cranial defect was analyzed grossly and then explanted for micro-CT, histologic, and biomechanical analyses.