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Functionalization of scaffolds with chimeric anti-BMP-2 monoclonal antibodies for osseous regeneration

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ABSTRACT

Recent studies have demonstrated the ability of murine anti-BMP-2 monoclonal antibodies (mAb) immobilized on an absorbable collagen sponge (ACS) to mediate *de novo* bone formation, a process termed antibody-mediated osseous regeneration (AMOR). The objectives of this study were to assess the efficacy of a newly generated chimeric anti-BMP-2 mAb in mediating AMOR, as well as to evaluate the suitability of different biomaterials as scaffolds to participate in AMOR. Chimeric anti-BMP-2 mAb was immobilized on 4 biomaterials, namely, titanium microbeads (Ti), alginate hydrogel, macroporous biphasic calcium phosphate (MBCP) and ACS, followed by surgical implantation into rat critical-size calvarial defects. Animals were sacrificed after 8 weeks and the degree of bone fill was assessed using micro-CT and histomorphometry. Results demonstrated local persistence of chimeric anti-BMP-2 mAb up to 8 weeks, as well as significant *de novo* bone regeneration in sites implanted with chimeric anti-BMP-2 antibody immobilized on each of the 4 scaffolds. Ti and MBCP showed the highest volume of bone regeneration, presumably due to their resistance to compression. Alginate and ACS also mediated *de novo* bone formation, though significant volumetric shrinkage was noted. *In vitro* assays demonstrated cross-reactivity of chimeric anti-BMP-2 mAb with BMP-4 and BMP-7. Immune complex of anti-BMP-2 mAb with BMP-2 induced osteogenic differentiation of C2C12 cells *in vitro*, involving expression of RUNX2 and phosphorylation of Smad1. The present data demonstrated the ability of chimeric anti-BMP-2 mAb to functionalize different biomaterial with varying characteristics to mediate osteogenesis.

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1. Introduction

Bone tissue reconstruction is often necessitated by congenital anomalies, infection, trauma, and skeletal diseases [1,2]. Bone tissue engineering concepts have sought to leverage advances in development of biomaterial scaffolds, signaling molecules and progenitor cells to regenerate tissues that match the physical and biological properties of natural tissues [3–5]. Multiple bone tissue engineering strategies have been reported in the literature, including acellular scaffolds, gene therapy, stem cell therapy, application of growth factors, and combinations of these strategies. Growth and differentiation factors such as bone morphogenetic proteins (BMPs), platelet-derived growth factors (PDGFs) and insulin-like growth factors (IGFs) have shown promising regenerative properties when applied to bone tissue engineering [6–10]. Studies have demonstrated that BMP-2, BMP-4 and BMP-7 have the ability to stimulate osteoprogenitor differentiation into mature osteoblasts. Pre-clinical and clinical studies have confirmed the osteoinductive potential of BMPs, leading to the FDA approval of recombinant human (rh) BMP-2 and rhBMP-7 as biologic agents used in regenerative medicine [11–14]. However, the clinical applications of these recombinant growth factors are limited due to several disadvantages including their high cost and the fact that super-physiologic doses are needed for bone formation (milligrams versus pictograms), their short *in vivo* half-life and their lower biologic activity than their endogenous counterparts [15–17].

An alternative to the administration of exogenous rhBMP-2 to induce bone regeneration is immobilizing antibodies (Abs) specific for BMP-2 on a solid scaffold and implanting this construct in the

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area where bone growth is desired in order to attract endogenous BMP-2 [18,19]. The application of Abs as therapeutic agents in bone tissue engineering was first reported by Freire et al., demonstrating the possibility of capturing endogenous BMP-2 to induce osteogenic differentiation of osteoprogenitor stem cells and *de novo* bone formation. This approach was termed antibody-mediated osseous regeneration (AMOR). However, previous studies have utilized murine-derived monoclonal antibodies in their studies [18,19]. Murine monoclonal antibodies are derived entirely from mice using hybridoma technology [20]. In humans, these murine antibodies often have limited clinical application due to their short circulating half-lives, their immunogenic nature and potential for adverse reactions including human immune effector responses [21,22]. Therefore, the present study was conducted to investigate the possibility of utilizing chimeric monoclonal antibodies (mAbs) as an alternative to murine antibodies in AMOR. Additionally, in their previous experiments Freire et al. immobilized murine mAbs on an absorbable collagen sponge (ACS), which is an FDA-approved, convenient scaffold for the application of rhBMP-2. However, ACS has low mechanical strength and its chemistry may be less than optimal, so there is impetus to explore alternative scaffolds with more desirable physical and chemical properties. Therefore, the main objectives of this study were twofold: first, to investigate the possibility of using chimeric anti-BMP-2 mAb for AMOR using both *in vitro* assay and an *in vivo* animal model; and second, to test different biomaterials as scaffolds for use in AMOR to immobilize chimeric anti-BMP-2 mAb. The following biomaterials were tested in our *in vivo* rat critical size calvarial model: alginate hydrogel, titanium microbeads, macroporous biphasic calcium phosphate (MBCP) bioceramic and ACS.

2. Materials and methods

2.1. Antibodies

The hybridoma clone of a murine anti-BMP-2 mAb (3G7, Abnova Inc., Taiwan) was expanded in non-selective hybridoma medium (Invitrogen, Carlsbad, CA), total RNA was purified, and mRNA coding for the immunoglobulin genes were purified using the Oligotex mRNA Kit (QIAGEN Inc., Chatsworth, CA). The mRNA was utilized to synthesize total complementary DNA (cDNA), which was subsequently amplified using PCR to yield light chain and heavy chain variable regions. After amplification, the PCR products of the variable regions were cut with restriction endonucleases *SalI* and *EcoRI* (New England Biolabs Inc., Ipswich, MA) for the heavy chain and *SalI* and *BamHI* (New England Biolabs Inc.) for the light chain. The cut variable regions were individually ligated into pBluescript plasmids (SK^+, Invitrogen), and the variable region genes were amplified from the pBluescript vectors via PCR using oligonucleotide primers designed to introduce appropriate restriction endonuclease sites and the Kozak translation initiation sequence. Specifically, *Hind3* and *BsiWI* (New England Biolabs Inc.) restriction sites were introduced for the light chain variable gene and *XbaI* and *NheI* restriction sites were added for the heavy chain variable gene. The light chain variable regions were ligated into the parent expression vector, into which the human kappa constant region had already been cloned. The heavy chain variable region was ligated into the parent GS expression vector, into which the human gamma 4 constant region had already been cloned. The final expression vectors contained transcription cassettes for the chimeric light and heavy chains, respectively.

The chimeric antibody was then expressed by NS0 cells (Invitrogen) using plasmid technology, and high-expressing subclones of chimeric mAb were placed in liquid suspension culture using selective medium containing 3% dialyzed fetal calf serum (Invitrogen) and penicillin and streptomycin antibiotics (Invitrogen). The cells were expanded to produce sufficient quantities of antibodies for subsequent testing. After 7 days of aeration (two weeks in culture), spent cultures were filtered through 0.2 μm filter units (Sartorius TCC Company, CO) and purified by tandem protein A affinity chromatography and ion exchange chromatography to yield antibody products with greater than 98% purity. Antibody was collected in PBS and syringe-filtered (Millipore, Billerica, MA) into sterile 5 mL glass vials for use in this study.

2.2. Flow cytometry

A flow cytometric assay was developed in order to study binding of the BMP-2 cellular receptor with the immune complex formed between chimeric anti-BMP-2 mAb and BMP-2, 4 and 7. Briefly, rhBMP-2, 4 and 7 (all 100 ng/mL, Medtronic, Minneapolis, MN) were incubated with chimeric mAb (25 μg/mL) for 30 min at 4 °C. The resultant immune complexes were then incubated with C2C12 cells (American Type Culture Collection, Manassas, VA), which express BMP-2 receptors. Subsequently, the immune complexes were immunolabeled using phycoerythrin-conjugated goat anti-human Ab (Santa Cruz Biotechnology, Dallas, TX). The intensity of fluorescent labeling was determined by measuring mean fluorescent intensity (MFI) using a flow cytometer (FACS Calibur; Becton Dickinson, Laguna Hills, CA). Controls included cells alone (−) and substitution of anti-BMP-2 mAb with isotype-matched mAb with no specificity (isotype mAb).

2.3. Cell culture

A mouse myoblast cell line (American Type Culture Collection) was used in this study. C2C12 cells were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin (Sigma–Aldrich), and 10% fetal bovine serum (Biocell Laboratories, Rancho Dominguez, CA) at 37 °C in a humidified atmosphere supplied with 5% CO_2.

2.4. In vitro osteogensis

In order to study the effects of the chimeric anti-BMP-2 mAb/BMP-2 immune complex on osteogenesis, an *in vitro* assay was utilized accordingly to a recently published method [34]. Briefly, the C2C12 myoblast cell line was selected since it does not express BMP-2. Accordingly, 25 μg/mL anti-BMP-2 mAb or isotype control mAb with no specificity (isotype mAb, negative control) was incubated in 6-well plates for 1 h at room temperature, followed by three washes and blocking with PBS containing 0.5% BSA (Invitrogen) to block unoccupied binding sites on the plastic surfaces. Plates were then incubated with sub-osteogenic concentrations (100 ng/mL) of BMP-2 for 1 h at room temperature followed by six washes to remove any unbound BMP-2 from the solution. Triplicate cultures of 1×10^5 C2C12 cells were grown in α-DMEM (Invitrogen) culture media containing 10% FBS (Biocell Laboratories), 1% penicillin and streptomycin. Positive control wells contained BMP-2 (100 ng/mL) in solution. Alizarin red S staining was utilized to assess osteogenic differentiation of C2C12 cells. Briefly, cell cultures were washed twice with PBS, fixed for 1 min in 60% isopropanol and rehydrated with distilled water. Cultures were then stained with Alizarin red S solution for 5 min, followed by two washes with deionized water. Positively stained cells were visualized under a microscope and quantified using NIH ImageJ software (NIH, Bethesda, MD).

Western blot analysis was performed after two weeks of culturing in the abovementioned conditions. Briefly, the cells were washed twice with PBS and lysed with protein extraction buffer for 30 s (BIO-RAD, Irvine, CA). After centrifugation, the supernatant solution was collected, and protein concentrations were determined using a BCA assay (Pierce, Rockford, IL). Equal amounts of protein extracts were fractionated in 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). These nitrocellulose membranes were incubated with mouse mAbs against p-Smad1 (Santa Cruz Biotechnology), Smad6 (Abcam, Cambridge, MA), Runx2 (Abcam) and the housekeeping gene beta-actin (Abcam).

2.5. Scaffold biomaterials

Alginate hydrogel (NovaMatrix FMC Biopolymer, Norway), macroporous biphasic calcium phosphate (MBCP, Hydroxyapatite/β-Tricalcium phosphate 20/80, Biomatlante, Vigneux de Bretagne, France), grade IV Titanium microbeads with 250 μm diameter (Sybron Dental Implants, Orange, CA) and ACS (Helicote, Miltex, Plainsboro, NJ) were used in this study. Chimeric anti-BMP-2 mAb (25 μg/mL) and isotype control mAb (25 μg/mL) were immobilized on each scaffold material, as previously described [18].

2.6. In vivo calvarial defect model

Two-month-old virgin female Sprague–Dawley rats ($n = 32$, Harlan Laboratories, Livermore, CA) were housed at 22 °C under a 12 h light and 12 h dark cycle and fed *ad libitum* (Purina Inc., Baldwin Park, CA). All animals were treated according to the guidelines and regulations for the use and care of animals at the University of Southern California. Calvarial defects were created in 8-week-old rats under general anesthesia. Full-thickness skin flaps were raised, exposing the left and right parietal bones. Defects in the midline of the parietal bone, 7 mm in diameter, were generated using a trephine under copious saline irrigation. Scaffold materials, each containing 25 μg/mL of mAb, were placed inside the calvarial defects. At the end of the treatment period, the animals were sacrificed in a CO_2 chamber and the skulls were harvested and stored in buffered formalin while awaiting analysis.

2.7. Histological and histomorphometric analysis

For histological analysis, the retrieved specimens were fixed with 4% (v/v) paraformaldehyde for 30 min at room temperature and placed in PBS for 15 min prior to dehydration. Serial dehydration was achieved by placing specimens in a sequential series of increasing ethanol concentrations to remove all of the water. The