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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.

1. Introduction

Bone tissue reconstruction is often necessitated by congenital anomalies, infection, trauma, and skeletal diseases [1]. 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

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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 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 *Sall* and *EcoRI* (New England Biolabs Inc, Ipswich, MA) for the heavy chain and *Sall* 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 μ m 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 μ g/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 Biotchnology, 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% CO₂.

2.4. In vitro osteogenesis

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 μ g/ml anti-BMP-2 mAb or isotype control mAb with no specificity (isotype mAb, negative control) was incubated in 6-well plates for one hour 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 hour 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 sec (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-Smad 1 (Santa Cruz Biotechnology), smad 6 (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/ -Tricalcium phosphate 20/80, Biomatlante, Vigneux de Bretagne, France), grade IV Titanium microbeads with 250 μ m diameter (Sybron Dental Implants, Orange, CA) and ACS (Helicote, Miltex, Plainsboro, NJ) were used in this study. Chimeric anti-BMP-2 mAb (25 μ g/mL) and isotype control mAb (25 μ g/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 μ g/mL of mAb, were placed inside the calvarial defects. At the end of the treatment period, the animals were sacrificed in a CO₂ 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 ethanol was then completely replaced with a series of solutions containing increasing concentrations of xylene, culminating in 100% xylene, prior to incubation with paraffin-saturated xylene at room temperature overnight. The specimens were then serially sectioned (6 μ m) and affixed to glass slides. Additionally, the paraffin was completely removed by immersion in xylene, followed by immersion in decreasing ethanol concentrations and then washing with tap water. The specimens containing titanium microbeads were imbedded in resin mounting solution and sectioned using Exakt grinder (Exakt 312, EXAKT, Germany). The sections were stained with hematoxylin and eosin (H&E). Images were captured using an Olympus

DP50 digital camera (Olympus Optical Co, Japan) and analyzed using Analysis imaging software (Soft Image System GmbH, Germany).

2.8. Micro-CT analysis

Specimens were scanned using a high-resolution micro-CT system (MicroCAT II, Siemens Medical Solutions Molecular Imaging, Knoxville, TN) for evaluation of ectopic mineralization, with scans of 10 μm width at 60 kV and 110 μA at a spatial resolution of 18.7 μm (Voxel dimension). Bone volume fraction (BV/TV) was calculated.

2.9. Confocal laser scanning microscopy (CLSM)

The concentration of the remaining immobilized chimeric mAb on each of the previously-implanted scaffolds was analyzed using CLSM (Fluoview FV10i; Olympus Optical Co). The samples were retrieved after 0, 1, 14 or 56 days of implantation, fixed with 4% PFA, incubated for 30 min at room temperature, washed, blocked with BSA and stained with secondary antibody (FITC-conjugated goat anti-Human IgG Ab), after which CLSM and analysis was performed. Additionally, in order to confirm the specificity of our chimeric anti-BMP-2 mAb, the specimens after one week of implantation were immunolabeled with donkey anti-goat IgG-FITC antibody (Santa Cruz Biotechnology) as negative control (-). The fluorescence intensity was quantified by Spot analysis software (SPOT Imaging Solutions, Sterling Heights, MI) with the same fluorescence threshold.

2.10. Statistical analysis of data

Quantitative data were expressed as mean $\pm$ standard deviation (SD). A two-tailed Student's t-test and one-way analysis of variance (ANOVA), followed by Tukey's test at significance level of $\alpha = 0.05$, were used for the comparison of multiple sample means.

3. Results

3.1. Binding of anti-BMP-2 mAb/BMP immune complexes to cells

In view of the significant structural homology between BMP-2, -4 and -7, it was sought to determine whether chimeric anti-BMP-2 mAb cross-reacts with BMP-4 and BMP-7. Western blot analysis confirmed cross-reactivity between chimeric anti-BMP-2 mAb cross-reacts with BMP-4 and BMP-7 (data not shown). Since antibodies can potentially serve as antagonists and prevent interactions of ligands with their cellular receptor, it was important to determine whether the immune complexes formed by BMP-2, BMP-4 or BMP-7 and chimeric anti-BMP-2 mAb bind with the BMP-2 cellular receptor. To that end, 100 ng/ml of BMP-2, BMP-4 or BMP-7 were incubated with chimeric anti-BMP-2 mAbs (25 $\mu\text{g}/\text{mL}$) (Fig. 1a). The resultant immune complexes were incubated with C2C12 cells, followed by immunofluorescent labeling with fluorochrome-conjugated secondary Abs and flow cytometry to detect cells that bound to an immune complex. The results demonstrated that anti-BMP-2 mAb immune complexes with BMP-2, BMP-4, or BMP-7 were each able to bind to C2C12 cells at significantly higher level compared with isotype control mAb incubated with BMP-2, -4 or -7 (Fig. 1b).

3.2. In vitro osteogenic response of C2C12 cells to anti-BMP-2 mAb/BMP immune complex

An *in vitro* assay was developed to evaluate the ability of immobilized anti-BMP-2 mAb/BMP-2 immune complexes to mediate osteogenic differentiation of C2C12 cells. Cells were cultured under three different conditions: (1) rhBMP-2 added in culture media (positive control), (2) immobilized chimeric anti-BMP-2 mAb + rhBMP-2 immune complex and (3) immobilized isotype control mAb with no specificity substituted for anti-BMP-2 mAb (negative control). Osteogenic differentiation of C2C12 cells after 2 weeks of culture was

analyzed using Alizarin red staining. Positive control cultures, as well as those with immobilized chimeric anti-BMP-2 mAb + BMP-2 immune complex differentiated into Alizarin red-positive cells (Fig. 2a). In contrast, negative control cultures remained negative for Alizarin red (Fig. 2b). No statistically significant difference between positive control and experiment cultures were noted (Fig. 2b).

To investigate the intracellular pathways of signaling involved following binding of chimeric anti-BMP-2 mAb/BMP-2 immune complex to C2C12 cells, the expression of p-Smad1, p-Smad6 and Runx2 was investigated. Results in Figure 2c demonstrated high expression levels of osteoblast-specific molecules, including Runx2 and p-Smad1 in cells cultured in the presence of the anti-BMP-2 mAb + BMP-2 immune complex for two weeks. In contrast, a low expression level for a downregulator of osteogenesis (Smad 6) was observed (Fig. 2c).

3.3. In vivo AMOR in response to anti-BMP-2 mAb

To investigate the ability of chimeric anti-BMP-2 mAb to mediate AMOR *in vivo*, the calvarial defect model in rats was utilized. A dose response experiment was performed in order to determine the optimal dose of mAb for mediating bone formation. *De novo* bone formation was detected in calvarial defects implanted with as little as 1 µg/mL of chimeric anti-BMP-2 mAb (Fig. 3a and 3b). A dose of 25 µg/mL of mAb was sufficient for complete repair of calvarial defects. Based on this, as well as our previous data on murine anti-BMP-2 mAbs [19], 25 µg/mL was selected as the dosage of chimeric mAb for all subsequent experiments. To investigate the ability of various biomaterials to serve as scaffolds for the mAb, 25 µg/mL of chimeric anti-BMP-2 mAb was immobilized on each of 4 different scaffold materials and implanted in rat calvarial defects. To evaluate *de novo* bone formation within the defects, micro-CT and histological analyses were performed after 8 weeks. Micro-CT analysis (Fig. 4a) demonstrated significantly greater amounts of bone fill and repair in defects implanted with scaffolds containing anti-BMP-2 mAb than in defects implanted with scaffolds containing isotype matched mAb ($P < 0.05$). The micro-CT data revealed that MBCP and Ti microbeads generated the largest volume of new bone, while alginate hydrogel scaffold showed the smallest amount of bone regeneration (Fig. 4b).

Histological evidence supported the micro-CT observations. Staining with H&E and Trichrome (not shown) demonstrated a significant degree of bone fill within calvarial defects implanted with scaffolds containing adsorbed chimeric anti-BMP-2 mAb (Fig. 5a). The presence of vital bone with osteocytes in lacunae was detected with each of the scaffolds (Fig. 5a). The histomorphometric data (Fig. 5b) were in good correlation with quantitative micro-CT data, revealing that MBCP and Ti microbeads exhibited the greatest amounts ($p < 0.05$) of bone regeneration, while alginate hydrogel scaffold showed the smallest amount.

3.4. In vivo persistence of anti-BMP-2 mAb

To examine *in vivo persistence of* anti-BMP-2 mAb immobilized on various scaffold materials, mAb was immobilized on the 4 scaffolds and implanted in calvarial defects. Animals were sacrificed at 1, 14 or 56 days after implantation. Calvarial specimens were examined with CLSM, following immunofluorescent labeling with FITC-conjugated goat anti-human mAb, to visualize the chimeric anti-BMP-2 mAb. Results demonstrated that the local concentrations of chimeric anti-BMP-2 mAb, as well as isotype control mAb (data not shown) progressively decreased over time (Figs. 6a and b). During the first day following *in vivo* implantation, between 25 to 50% of the chimeric anti-BMP-2 was lost, depending on the biomaterial used as the scaffold (Fig. 6c). By Day 14, approximately 25% of the initial concentrations of chimeric anti-BMP-2 persisted on all 4 biomaterial. At 8 weeks, low levels

of chimeric anti-BMP-2 that was statistically higher than background were detectable ($p < 0.05$).

4. Discussion

BMPs have been identified as the major mediators in bone regeneration and formation. It is well known that exogenous administration of rhBMP-2 can initiate a healing cascade that induces bone regeneration through the TGF- β /BMP signaling pathway [23, 24]. BMP-2 is a member of the TGF- β family, assembles into a biologically active homodimer, which binds to heterodimeric type I and type II receptors for BMP-2 (Fig. 2d) [25]. Our laboratory has introduced AMOR as a tissue engineering strategy alternative to administration of exogenous growth factors. Since our ultimate objective is translational application of AMOR, we generated a chimeric anti-BMP-2 mAb to reduce the potential for xenogenic immune response to the mAb. One of the objectives of the present study was to determine whether application of the chimeric anti-BMP-2 mAb is associated with any adverse responses. Careful examination of histologic slides of calvaria treated with chimeric anti-BMP-2 mAb or isotype control mAb failed to find any evidence of significant inflammatory or pathologic changes. Detailed necropsy of major organs, as well as examination of serum for acute phase inflammatory markers also failed to find any significant aberrant systemic effects (manuscript in preparation). Previous studies with chimeric mAb's have generally shown their clinical safety profile [28,29]. There are currently eight chimeric therapeutic antibodies, which have been approved by US FDA and are being used clinically [29].

In view of the fact that the anti-BMP-2 mAb used in this study has been generated against a human BMP-2 immunogen, one of the questions was whether it will be efficacious in binding rat BMP-2 after *in vivo* implantation. The results of calvarial study clearly demonstrated the efficacy of chimeric anti-BMP-2 mAb in mediating *de novo* bone formation. This was not surprising, in light of the significant degree of homology (92.2%) between the human and rat BMP-2 genes [20]. Taking into account the high degree of homology of BMP-2 with other osteogenic BMPs such as BMP-4 and BMP-7, the binding capacity of chimeric anti-BMP-2 mAb with BMP-4 and BMP-7 was also examined. The cross-reactivity of murine anti-BMP-2 parent clone of our chimeric anti-BMP-2 mAb with BMP-4 and BMP-7 has previously been demonstrated [19]. Moreover, it has been reported that implantation of immobilized murine anti-BMP-2 mAb leads to *in vivo* binding of BMP-2, BMP-4 and BMP-7. Our *in vitro* assay confirmed the crossreactivity of chimeric anti-BMP-2 mAb with BMP-4 and BMP-7 [19]. A potential interpretation of these data with regards to the *in vivo* mechanism of anti-BMP-2 mAb action is that this antibody can capture at least three osteogenic BMPs. These BMPs can act in an additive or synergistic manner to induce *de novo* bone formation. Follow-up studies are pending to determine what other immunologically related osteogenic molecules are bound to the chimeric anti-BMP-2 mAb following *in vivo* implantation. The present study has documented some of the downstream events following engagement of the BMP receptor, including the expression of p-Smad 1 and Runx2. Moreover, the expression level of downregulators of osteogenesis (such as Smad 6), which play a critical role in the inhibition of the BMP signaling pathway, was apparently not affected.

In tissue engineering, biomaterials are increasingly utilized for sophisticated functions such as scaffolds to guide the development of new tissues and organs [26]. To direct cell and tissue response, biomaterial design has focused on controlling the material chemistry to promote highly specific binding interactions between the materials and surrounding cells [27, 28]. Here we studied the feasibility of using four different types of scaffolds with immobilized chimeric anti-BMP-2 mAb in AMOR. This modality of treatment involves implanting a scaffold impregnated with anti-BMP-2 mAb in a bone defect to capture

endogenous BMP-2, as well as other related osteogenic growth factors, which can induce bone regeneration. The ability of AMOR to regenerate bone may be attributed to the capacity of anti-BMP-2 mAb to capture and tether multiple endogenous osteogenic BMP's and therefore increase their local availability and persistence. Moreover, a robust cellular infiltration has been observed in sites implanted with anti-BMP-2 mAb [19]. The co-delivery of anti-BMP-2 mAb and mesenchymal stem cells has also been shown to lead to greater degree of bone repair in calvarial defects [34].

The current study confirmed the ability of chimeric anti-BMP-2 mAb immobilized on all four of the biomaterials studied to mediate robust *de novo* bone formation in rat calvarial defects, as confirmed by micro-CT and histological analyses. The bioceramic MBCP and titanium microbeads showed the largest volume of bone regeneration, while alginate hydrogel scaffold showed higher volumetric shrinkage. The differences between the volumes of bone regeneration may be attributed to the ability of the prospective scaffold to withstand compressive forces. In view of the fact that different skeletal defects present with varying physical requirements, it was important to test the feasibility of immobilizing chimeric anti-BMP-2 mAb on different types of scaffolds with different physical properties. Each of the scaffolds utilized have unique properties, which make them suitable for different applications. Titanium is used extensively in orthopedic [30] and dental implant [31] therapies, where anti-BMP-2 mAb can potentially be used for improving the efficacy of osseointegration. Alginate hydrogel can be formulated as an injectable scaffold [32] and has been used extensively in tissue engineering [33]. Therefore, alginate or ACS may be suitable for intraosseous defects, where it is desirable to have a biodegradable scaffold and compressive strength will not be required. MBCP is likely to have application in repair of osseous defects, which require resistance to compression. Current studies are in progress to investigate the physical mechanical properties of regenerated bone using each of the scaffolds functionalized with chimeric anti-BMP-2.

5. Conclusions

Here we report the application of chimeric anti-BMP-2 mAb immobilized on different types of scaffolds in the process of antibody mediated bone regeneration. The propose system has the ability to mediate *de novo* bone formation efficiently, as confirmed by our *in vitro* and *in vivo* studies. Furthermore, we report the cross-reactivity of the newly generated chimeric anti BMP-2 mAb with BMP-4 and BMP-7 through specificity studies. The anti-BMP-2 antibody is capable of binding to endogenous BMP-2, BMP-4 and BMP-7, leading to enhanced antibody-mediated bone regeneration. Finally, AMOR can be regulated through the BMP signaling pathway, as confirmed by the *in vitro* experiments.

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References

1. Sachlos E, Czernuszka JT. Making tissue engineering scaffolds work. Review on the application of solid freeform fabrication technology to the production of tissue engineering scaffold. *Eur Cell Mater.* 2003; 5:29–40. [PubMed: 14562270]
2. Lin NH, Gronthos S, Bartold PM. Stem cells and periodontal regeneration. *Aust Dent J.* 2008; 53:108–121. [PubMed: 18494965]
3. Chen FM, Zhang J, Zhang M, An Y, Chen F, Wu ZF. A review on endogenous regenerative technology in periodontal regenerative medicine. *Biomaterials.* 2010; 31:7892–7927. [PubMed: 20684986]

4. Monaco E, Bionaz M, Hollister SJ, Wheeler MB. Strategies for regeneration of the bone using porcine adult adipose-derived mesenchymal stem cells. *Theriogenology*. 2011; 75:1381–99. [PubMed: 21354606]
5. Porter JR, Ruckh TT, Popat KC. Bone Tissue Engineering: a Review in Bone biomimetics and drug delivery strategies. *Biotechnol Prog*. 2009; 25:1539–1560. [PubMed: 19824042]
6. Betz VM, Betz OB, Harris MB, Vrahas MS, Evans CH. Bone tissue engineering and repair by gene therapy. *Front Biosci*. 2008; 13:833–841. [PubMed: 17981592]
7. Arrington ED, Smith WJ, Chambers HG, Bucknell AL, Davino NA. Complications of iliac crest bone graft harvesting. *Clin Orthop Relat Res*. 1996; 329:300–309. [PubMed: 8769465]
8. Parfitt AM. The cellular basis of bone remodeling: the quantum concept re-examined in light of recent advances in the cell biology of bone. *Calcif Tissue Int*. 1984; 36:S37–S45. [PubMed: 6430521]
9. Jiang T, Abdel-Fattah WI, Laurencin CT. *In vitro* evaluation of chitosan/poly (lactic acid-glycolic acid) sintered microsphere scaffolds for bone tissue engineering. *Biomaterials*. 2006; 27:4894–4903. [PubMed: 16762408]
10. van den Dolder J, Farber E, Spauwen PH, Jansen JA. Bone tissue reconstruction using titanium fiber mesh combined with rat bone marrow stromal cells. *Biomaterials*. 2003; 24:1745–1750. [PubMed: 12593956]
11. Wikesjo U, Polimeni G, Qahash M. Tissue engineering with recombinant human bone morphogenetic protein-2 for alveolar augmentation and oral implant osseointegration: experimental observations and clinical perspectives. *Clin Implant Dent Relat Res*. 2005; 7:112–122. [PubMed: 15996358]
12. Knippenberg M, Helder MN, Zandieh DB, Wuisman PI, KleinNulend J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. *Biochem Biophys Res Commun*. 2006; 342:902–908. [PubMed: 16500625]
13. Chin M, Ng T, Tom W, Carstens M. Repair of alveolar clefts with recombinant human bone morphogenetic protein (rhBMP-2) in patients with clefts. *J Craniofac Surg*. 2005; 16:778–89. [PubMed: 16192856]
14. Khan S, Lane J. The use of recombinant human bone morphogenetic protein-2 (rhBMP-2) in orthopaedic applications. *Expert Opin Biol Ther*. 2004; 4:741–8. [PubMed: 15155165]
15. Oldham J, Lu L, Zhu X, Porter B, Hefferan T, Larson D, et al. Biological activity of rhBMP-2 released from PLGA microspheres. *J Biomech Eng*. 2000; 122:289–292. [PubMed: 10923299]
16. Ishikawa H, Kitoh H, Sugiura F, Ishiguro N. The effect of recombinant human bone morphogenetic protein-2 on the osteogenic potential of rat mesenchymal stem cells after several passages. *Acta Orthop*. 2007; 78:285–96. [PubMed: 17464620]
17. Chen D, Zhao M, Mundy G. Bone morphogenetic proteins. *Growth Factors*. 2004; 22:233–45. [PubMed: 15621726]
18. Freire MO, You HK, Kook JK, Choi JH, Zadeh HH. Antibody-mediated osseous regeneration: a novel strategy for bioengineering bone by immobilized anti-bone morphogenetic protein-2 antibodies. *Tissue Eng Part A*. 2011; 17:2911–2921. [PubMed: 21870943]
19. Freire MO, Kim HK, Kook JK, Nguyen A, Zadeh HH. Antibody-mediated osseous regeneration: the early events in the healing response. *Tissue Eng Part A*. 2013; 19:1165–74. [PubMed: 23190409]
20. Ober RJ, Radu CG, Ghetie V, Ward ES. Differences in promiscuity for antibody- FcRn interactions across species: implications for therapeutic antibodies. *Int Immunol*. 2001; 13:1551–1559. [PubMed: 11717196]
21. Tonegawa S. Somatic generation of antibody diversity. *Nature*. 1983; 302:575–581. [PubMed: 6300689]
22. Schroff RW, Foon KA, Beatty SM, Oldham RK, Morgan AC Jr. Human anti-murine responses in patients receiving monoclonal antibody therapy. *Cancer Res*. 1985; 45:879–885. [PubMed: 3871353]
23. Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res*. 2009; 19:71–88. [PubMed: 19002158]

24. Ishikawa H, Kitoh H, Sugiura F, Ishiguro N. The effect of recombinant human bone morphogenetic protein-2 on the osteogenic potential of rat mesenchymal stem cells after several passages. *Acta Orthop*. 2007; 78:285. [PubMed: 17464620]
25. Liu DD, Zhang JC, Zhang Q, Wang SX, Yang MS. TGF- β /BMP signaling pathway is involved in cerium-promoted osteogenic differentiation of mesenchymal stem cells. *J Cell Biochem*. 2013; 114:1105–14. [PubMed: 23150386]
26. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006; 126:677–689. [PubMed: 16923388]
27. Valamehr B, Jonas SJ, Polleux J, Qiao R, Guo S, Gschwend EH, et al. Hydrophobic surfaces for enhanced differentiation of embryonic stem cell-derived embryoid bodies. *Proc Natl Acad Sci U S A*. 2008; 105:14459–14464. [PubMed: 18791068]
28. Sapir Y, Kryukov O, Cohen S. Integration of multiple cell-matrix interactions into alginate scaffolds for promoting cardiac tissue regeneration. *Biomaterials*. 2011; 7:1838–47. [PubMed: 21112626]
29. Waldmann TA. Immunotherapy: past, present and future. *Nat Med*. 2003; 9:269–77. [PubMed: 12612576]
30. Brunette, DM.; Tengvall, P.; Textor, M. Properties and biological significance of natural oxide films on titanium and its alloys. Springer; 2001. *Titanium in Medicine*; p. 171-230.
31. Meffert RM, Langer B, Fritz ME. Dental implants: a review. *J Perio*. 1992; 63:859–870.
32. Alsberg A, Anderson KW, Albeiruti A, Franceschi RT, Mooney DJ. Cell-interactive alginate hydrogels for bone tissue engineering. *J Dent Res*. 2001; 80:2025–2029. [PubMed: 11759015]
33. Moshaverinia A, Chen C, Akiyama K, Ansari S, Xu X, Chee W, et al. Alginate as a potential scaffold for dental-derived stem cells: An *in vitro* study. *J Mater Sci: Mater Med*. 2012; 23:3041–51. [PubMed: 22945383]
34. Moshaverinia A, Ansari S, Chen C, Akiyama K, Xu X, Snead ML, et al. Coencapsulation of anti-BMP2 monoclonal antibody and mesenchymal stem cells in alginate microspheres for bone tissue engineering. *Biomaterials*. 2013; 34:6572–9. [PubMed: 23773817]

Figure 1.

(a) Schematic representation of the flow cytometric assay to study the binding ability of anti-BMP mAb + rhBMP-2 immune complexes to BMP-receptor positive cells. Anti-BMP-2 mAb and rhBMPs were incubated to form immune complexes, and the resultant complexes were followed by incubation of immune complexes incubated with C2C12 cells. The cell-bound immune complexes were detected with a fluorescently- conjugated secondary antibody and flow cytometric analysis. (b) Flow cytometric data examining binding of anti-BMP-2 mAb immune complexes with rhBMP-2, rhBMP-4 and rhBMP-7 to BMP-receptor-positive C2C12 cells. The mean fluorescent intensity (MFI) of flow cytometric analysis showed significant binding between anti-BMP-2 mAb and BMP-2, BMP-4 and BMP-7. Results confirmed the presence of binding interaction between the chimeric anti-BMP-2 and BMP-2, BMP-4 and BMP- 7. **, $p < 0.01$.

Figure 2.

(a) Alizarin red staining indicating mineralized nodule formation of cultured C2C12 cells after treatment with either BMP-2 or chimeric anti-BMP-2 mAb after four weeks. (b) Quantitative analysis of the amount of alizarin staining. (c) Western blot analysis showing the effect of chimeric mAb on the levels of expression of regulators of osteogenesis in C2C12 cells. (d) Graphical summary of interactions between chimeric anti-BMP-2 mAb and the receptors (BMPR-I and BMPR-II) that mediate AMOR through the BMP signaling pathway. NS: not significant.

Figure 3.

Dose response study of chimeric anti-BMP-2 mAb adsorbed on ACS implanted in rat calvarial defects. Representative 3D reconstruction of micro-CT images (a) and histomicrographs (b) of rat calvarial defects 8 weeks after implantation with different concentrations of chimeric anti-BMP-2 mAb adsorbed on ACS (scale bar = 1 mm). (c) Quantitative analysis of micro-CT data, expressed as % bone fill within calvarial defects. (d) Histomorphometric analysis of Trichrome-stained sections in panel b, expressed as % osteoid bone. Each concentration was compared to isotype mAb as the negative control. Anti-BMP-2 mAb at 25 µg/mL and rhBMP-2 showed the largest amounts of new bone formation. *, Asterisk symbols show the groups that are significantly different ($p < 0.05$).

Figure 4. (a) Representative 3D reconstruction of micro-CT images of rat calvarial defects 8 weeks after implantation with chimeric anti-BMP-2 mAb adsorbed on 4 different scaffold materials. (b) Quantitative analysis of micro-CT data, expressed as % bone fill within calvarial defects. **, $p < 0.01$.

Figure 5.

(a) Histological analysis of rat calvarial bone defects implanted with chimeric anti-BMP-2 mAb immobilized on 4 different types of scaffolds (scale bar = 1 mm for low magnification images and 50 μ m for histomicrographs in high magnification). (b) Quantitative histomorphometric analysis performed on H&E-stained sections showing percentage of new bone formation. **, $p < 0.01$.

Figure 6.

Confocal laser scanning microscopy analysis of scaffolds functionalized with anti-BMP-2 mAb retrieved at different time points. Representative CLSM images (a) and quantitative analysis (b, c) of immunofluorescence of 4 different biomaterials with immobilized anti-BMP-2 mAb. Calvarial specimens were retrieved at 1, 14 and 56 days and immunofluorescently labeled with FITC-conjugated goat anti-Human IgG Ab and imaged by CLSM. To control for non-specific labeling, controls were immunolabeled with donkey anti-goat IgG-FITC. (b) Quantitative analysis of fluorescence intensity. (c) Reduction in the fluorescence intensity for each scaffold from day 0 to day 56 after implantation. Three specimens for each group were tested. * $p < 0.05$.