Repair of large cranial defects by *hBMP-2* expressing bone marrow stromal cells: Comparison between alginate and collagen type I systems

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Abstract: Despite a wide range of available sources for bone repair, significant limitations persist. To bioengineer bone, we have previously transferred adenovirus-mediated human *BMP-2* gene into autologous bone marrow stromal cells (MSC). We have successfully repaired large, full thickness, cranial defects using this approach. We report now the effectiveness of various hydrogels as the scaffold for this type of bone regeneration, comparing specifically alginate with Type I collagen. Cultured MSC of miniature swine were infected with *BMP-2* or β-*gal* adenovirus 7 days before implantation. These cells were mixed with alginate, ultrapure alginate, alginate-RGD, or type I collagen to fabricate the MSC/biomaterial constructs. The results of cranial bone regeneration were assessed by gross examination, histology, 3D CT, and biomechanical tests at 6 weeks and 3 months after implantation. We found that the *BMP-2* MSC/collagen type I construct, but not the β-*gal* control, effectively achieved nearly complete repair of the cranial defects. No bone regeneration was observed with the other hydrogels. Biomechanical testing showed that the new bone strength was very close and only slightly inferior to that of normal cranial bone. Controlling for the integration of stem cells and *ex vivo* gene transfer, the alginate scaffolds has a significant negative impact on the success of the construct. Our study demonstrates better bone regeneration by collagen type I over alginate. This may have therapeutic implications for tissue engineered bone repair. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res 94A: 433–441, 2010

Key words: alginate; collagen type I; bone marrow stromal cells; BMP-2

INTRODUCTION

Over one million surgical procedures in the United States each year involve bone or cartilage substitutes.[1] The autogenous vascularized bone grafts, also known as free flaps, may be harvested from several areas of the body including temporal, scapular, radial, rib, iliac, fibular, or metatarsal bones. These grafts have functioned well but are associated with the donor site morbidities. The application of these flaps as allografts is limited due to rejections, infectious disease transmission, premature resorption, and donor shortage. Another alternative is to induce new bone formation. However, the new bone produced from inductive peptides (rhBMPs),[2] demineralized bone powder,[3] or mixtures of both[4] are usually limited in size. Biocompatible bone substitutes[5] have not functioned reliably in defects of critical size. In recent years, the advances in tissue engineering have made the creation of functional tissues possible by employing biocompatible and biodegradable biomaterials as scaffolds seeded with live cells.[1] The earlier work has demonstrated that implantation of osteoblast-PGA/PLA constructs into animal hosts successful could result in new bone formation with a final morphology similar to that of the initial polymer scaffold.

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Alginate is a natural anionic polysaccharide widely used in cell culture and drug delivery,[6] and its application relies upon the sol/gel transition in the presence of multivalent cations, such as Ca^{2+}. Sodium alginate has been used as a material for the encapsulation and immobilization of various cell types for immune-isolation and biochemical processing applications. Alginate cross-linked with calcium can act as a substrate for rat marrow cell proliferation and has potential for use as a 3D degradable scaffold.[7] Alginate has been previously used for cartilage and bone tissue engineering[8,9] and its uses in long-term culture of osteocytes and chondrocytes have been extensively documented.[10,11] Alginate has also been successfully used in *in vivo* bone formation. It has the advantage of being injectable and thus can be introduced via syringe to deliver a cell-alginate mixture, gel, or a calcium alginate film.[10,12–14] The development of the new ultrapure form of alginate (Pronova) makes an autologous model a real clinical possibility.[15]

Cell-adhesion ligands such as arginine-glycine-aspartic acid (RGD) can be engineered to the alginate polymer with improved cell adhesion.[16,17] Such RGD-alginate hydrogel with incorporated growth factors improved the healing and stability of 8 mm segmental bone defect stability by 16 weeks after implantation in nude mice.[12] Cells within RGD-modified alginate microspheres were able to establish more interactions with a synthetic extracellular matrix and demonstrated a much higher level of differentiation when compared to cells immobilized within unmodified alginate microspheres.[18] Taken together, these findings demonstrate that peptides covalently coupled to alginate can influenced cell behavior, and could adequately induce osteoblastic differentiation after MSC transplantation.[18]

Collagen, the most abundant protein in the body and the major component of the extracellular matrix, is regarded as one of the most useful biomaterials for growth factor delivery owing to its excellent biocompatibility and safety.[16] An adsorbed collagen type I coating stimulated the osteoblastic differentiation of rat bone marrow cells,[19] and collagen type I increased bone remodeling around hydroxyapatite implants in the rat tibia.[20]

Osteoblasts are considered to be derived from pluripotential mesenchymal stem cells, which develop into various cellular lineages capable of producing bone, muscle, cartilage, adipose, tissue, and fibrous tissues.[21,22] The ultimate fate of an undifferentiated mesenchymal stem cell is largely determined by its local environment,[23] and thus the use of inducing factors to stimulate differentiation has been of special interest.[24–28] Notably, bone morphogenetic protein (BMP) has been shown to successfully induce the development of osteoblasts from mesenchymal stem cells.[29]

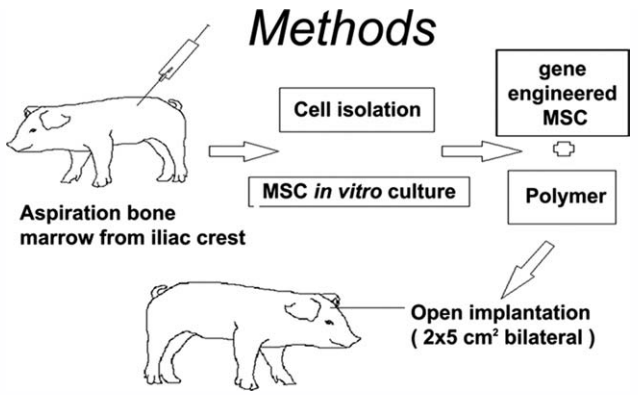

Figure 1. Bone marrow stromal cells (MSC) were aspirated from the iliac crest, separated, cultured, and passaged for 1 month in order to obtain adequate cell numbers. Cells used for implantation at the experimental sites and control sites were infected with adv-BMP-2 and adv-βgal, respectively. Biomaterials were mixed with MSC at a concentration of 50 million cells/mL.

We have previously demonstrated that undifferentiated mesenchymal stromal cells (MSC) infected with an adenovirus containing the *BMP-2* gene could promote osteoblast differentiation and repair critical size craniofacial bone defects in miniature swine.[30,31] To further improve this process by optimizing the scaffolds, we conducted the current study to investigate whether the use of various types of alginate, ligands-associated alginate, or collagen type I could facilitate and/or enhance bone formation during the repair of cranial defects in swine.

MATERIALS AND METHODS

All procedures involving animals were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chang Gung Memorial Hospital.

The aspirates from miniature swine iliac crest were prepared and processed as shown in Figure 1. The bone marrow stromal cells (MSC) were isolated and propagated up to a cell density of 50 million cells/mL. Cranial defects were generated with complete removal of the osteogenic periosteum and dura. MSC infected with adenovirus containing BMP-2 gene loaded into biomaterial constructs were implanted (Fig. 2).[31]

Construction of recombinant adenovirus

Adenovirus BMP-2, a replication-defective adenovirus vector containing the human *BMP-2* gene, was constructed as previously described.[30] The recombinant adenovirus contains the human *BMP-2* gene under the transcriptional control of the cytomegalovirus early gene promoter/enhancer.[31] The recombinant adenovirus containing the

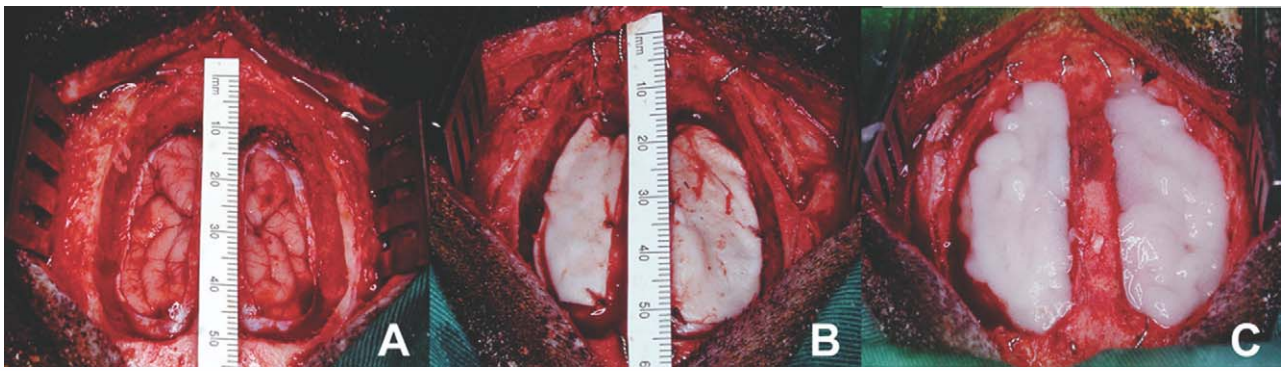

Figure 2. Generation of the cranial defects (2 × 5 cm^2) in miniature swine. (A) Bilateral bony defect, underlying dura removed. (B) Pig alloderm used for dura repair. (C) Defects filled with adenovirally infected MSC/biomaterial constructs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bacterial β-*galactosidase* (*lac z*) gene (designated as adenovirus β-*galactosidase*) was also constructed by the same strategy.[32]

Bone marrow aspiration and adenoviral infection

Forty miniature swine (Mitsae Pig, CGU, Taiwan) were randomly assigned to 4 groups of 10, each group treated with a separate polymer construct: group I, ultrapure alginate (Ultrapure MVG, Pronova Biopolymer, Drammen, Norway); group II, alginate (Sigma Chemical, St. Louis, Mo); group III, alginate RGD (a gift from Professor David J. Mooney) and group IV, collagen type I (Pancogene S, Gattefosse, St. Priest, Cedex, France) (Table I).

To prepare bone marrow aspirates, miniature swine were anesthetized by an intramuscular injection of 2% rompum (Bayer; 1 mL/10 kg) and ketamine (50 mg/mL, Yung Shin Pharmaceutical Industrial, Taiwan). Aspirated bone marrow (20 mL) was mixed with 2 mL of heparin sodium (5000 unit/mL, Agglutex, China Chemical & Pharmaceutical, Taiwan). Five to ten milliliters of aspirated iliac crest marrow was transferred to sterile tubes, to which 20 mL of complete medium was added. The mixtures were centrifuged at 1000 rpm for 5 min, and the supernatant and fat layers were removed. The cell pellets (2.5–5.0 mL) were resuspended, loaded onto 70% Percoll (Sigma, St. Louis, Missouri) gradients and centrifuged again at 460 g for 15 min. Three fractions were harvested: the top 25% contained low density cells, at 1.03 g/mL pooled density; the middle 50% contained high density cells of 1.10 g/mL pooled density; and the bottom 25% contained the cells of the highest density, at 1.14 g/mL pooled density. In preliminary experiments, each of these three pools was plated separately in complete medium in 100-mm dishes. Adherent marrow-derived mesenchymal cells were detected within the low-density fraction. Thus, the low-density fraction was the source for the adherent cell cultures for the subsequent experiments. The culture medium was changed every 3–4 days, and after 1 month the culture reached a cell density of ~50 million/mL.

Adenovirus-mediated human *BMP-2* gene transfer to MSC was performed 7 days before the generation of cranial defects. Adenovirus-mediated β-*Gal* gene transfer to MSC was performed as a control and in parallel. Infected cells were then trypsinized with 0.25% trypsin, washed 3 times, and cell numbers were determined with a hemocytometer. Cells (5 × 10^7 mL^{-1}) were then mixed separately with one of the four different polymer scaffolds.

Creation of cranial bony defects and implantation

All miniature swine were intubated and kept on ventilators. Two cranial defects (2 × 5 cm^2 each) were created per animal. The periosteum (from above), the dura (from below) and the cranium bone were completely removed to ensure that no osteoinductive tissue remained [Fig. 2(A)]. The dura defects were repaired with acellular dermis (Pig Alloderm, LifeCell) [Fig. 2(B)].

Six milliliters of cell/polymer constructs were implanted at each defect site [Fig. 2(C)]. The adenovirus-mediated *BMP-2* gene transferred MSC/polymer construct was used to fill right side cranial defect, and the β-*Gal* gene trans-

TABLE I
The Layout of Four Different Groups Each with Right and Left Defects and Different Treatment, and Each Group with Two Different Time Points

Group	I		II		III		IV	
Implantation period	6w	3M	6w	3M	w	3M	6w	3M
Adv-βgal (L) Adv-hBMP (R)	Ultra pure alginate		alginate		alginate RGD		collagen type I	

Cranial defect site: L = left, R = right.

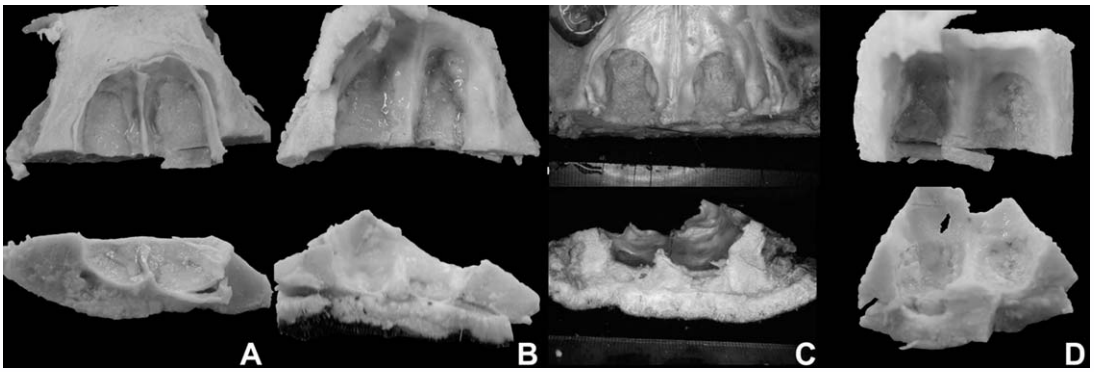

Figure 3. Gross morphology of the cranium after 3 months of *in vivo* implantation. Top row: Cranium view. Bottom row: Transverse view. Each panel, left: BMP-2 site, right: βgal site. (A) Group I (alginate, ultrapure); (B) group II (alginate); (C) group III (alginate RGD); (D) group IV (collagen).

ferred MSC/polymer construct was used to fill left side defect. The scalp wounds were closed in a watertight manner.

Alginate processing

Isolated cells were resuspended in a 2% sterile sodium alginate solution (0.1*M* K_2HPO_4, 0.135*M* NaCl, pH 7.4), which had previously been sterilized by a 0.45-nm filter, to yield a cellular concentration of 5×10^7/mL alginate solution. Immediately before implantation, sterilized $CaSO_4$ (0.2 g/mL of alginate) in PBS solution was mixed with cell-alginate construct to initiate gel formation. The cell/alginate/$CaSO_4$ mixture was delivered to defects using a 10-mL syringe and an 18.5-gauge needle. Groups I, II, and III gelled ~10 min after mixing.

Collagen processing

For the collagen group, a 4 mg/mL collagen solution (Pancogen S, Gattefosse Cedex, France) was dialyzed in diluted HCl solution under sterile condition for 8 days. Immediately before implantation, the cell/collagen mixture was delivered to defects using a 10-mL syringe and an 18.5-gauge needle. Group IV (collagen) gelled immediately after mixing.

To prevent potential mixing of the different constructs, an intact central bridge of bone, measuring 0.5×5 cm^2, was maintained and a watertight closure was made over each of the two lateral test defects.

Histological examination

Samples harvested from the cranial defects

Five miniature swine from each group were sacrificed at 6 weeks, and five were sacrificed at 3 months after implantation. Harvested samples from the repaired cranium bones were fixed in buffered 10% formalin for 72 h and sawed into two halves. One half was decalcified in Decalcifier I solution (Surgipath, Northbrook, IL) for 48 h. The specimen was then embedded, sectioned, and stained with hematoxylin and eosin. The nondecalcified half was stained with von Kossa's silver nitrate to analyze matrix mineralization.[33]

3D CT imaging

The G. E. Prospeed Plus Model: Sycal 800 (General Electic, Yokogawa Japan) used in this study allowed a 3 mm thickness and interval to achieve retro-reconstruction. One and one-half mm 3D CT images of the cranium were performed prior to the biomechanical and histology study. The regenerated bone areas were calculated by Analyser® 4.0 software (Biomedical Imaging Resource, Mayo Foundation, Rochester, Minn.).

Biomechanical analysis

The samples were stored at −70°C before testing. All specimens were machined and finished to 9 mm in diameter and 2 mm in height, and used to measure the ultimate compressive strength (Fig. 3). Each specimen was tested to failure in axial compression using an Instron testing machine (Model: 5544, Instron, Carton, Ma). To ensure perpendicular compression, a 10-mm diametric cylindrical rod with self-aligned function was used as the plunger, which was clamped on the upper side of a custom designed grip connecting to a 500 N load cell. The specimen was then placed on a flat supporting jig clamped on the lower side of the Instron frame. After the specimen was positioned, the compressive force was applied at a constant crosshead rate of 2 mm/min and the relation between force and displacement was recorded in 0.05 mm increments by the Instron Merlin software. The magnitude of the ultimate force of each individual specimen was selected for comparison. The testing conditions of the ultimate compressive test are listed as follows: displacement control mode, 2 mm/min in crosshead rate, 500 N as maximum capacity, and data acquisition at 1 datum/0.05 mm.

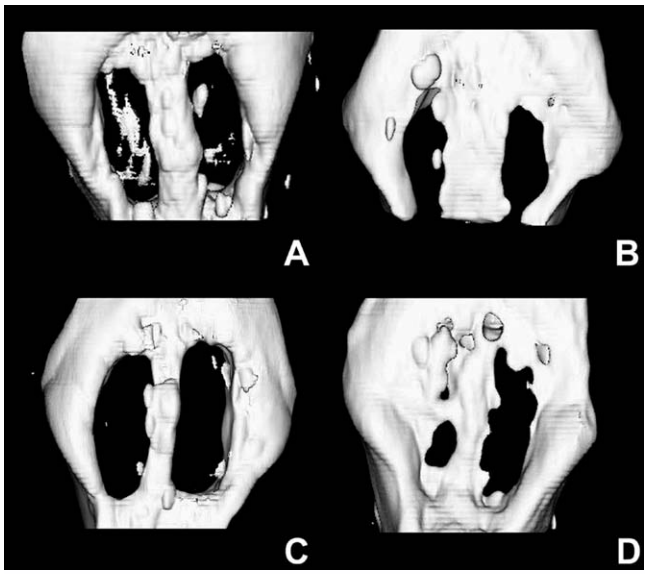

Figure 4. 3D CT imaging at 3 months. Each panel, left: BMP-2 site, right: βgal site. (A) Group I (alginate, ultrapure); (B) group II (alginate); (C) group III (alginate RGD); (D) group IV (collagen).

Statistical analysis

All measurements were collected in triplicate and expressed as means ± standard deviations. Single factor analysis of variance (ANOVA) was employed to assess the statistical significance of results for all the 3D CT images of the repaired bone areas.

RESULTS

Cranial defects repair

Gross bone formation was absent or incomplete after 3 months at both cranial defect sites implanted with adenovirus-mediated *BMP-2* transferred MSC and β-*Gal* transferred MSC in groups I, II, and III [Fig. 3(A–C)]. In contrast, in group IV (type I collagen), there was noticeable white bone formation at the site filled with *BMP-2* infected cells, compared to a lack of obvious bone formation at the control site implanted with β-*Gal* cells [Fig. 3(D)]. In all groups, the dura had completely healed and was detachable from the construct.

3D CT image analysis

Consistent with the results shown in Figure 3, the 3CD CT analysis of the cranial defects revealed that there were little or no new bone formations in *BMP-2* or β-*Gal* MSC implants in groups I, II, and III [Fig. 4(A–C)]. In contrast to the other groups and confirming previous observations, the 3D CT image of group IV demonstrated more bone formation and repair of the defect area implanted with *BMP-2* MSC, but not control cells [Fig. 4(D)].

Samples harvested 6 weeks after implantation from all groups and evaluated by 3D CT imagining showed no significant differences in bone formation between the control and the test defects. However, 3 months after implantation, a significant increase of bone formation at the defect site reconstructed by *BMP-2* infected MSC in group IV was clearly detected (Table II).

Histological observation

H&E staining did not reveal any significant bone formation at either cranial defect site in Groups I, II, and III. However, the cranial defect site implanted with *BMP-2* expressing MSC in Group IV showed characteristics of cancellous bone [Fig. 5(A)], and strongly positive Von Kossa staining at 3 months, indicating good mineralization; no evidence of cortical bone formation was observed [Fig. 5(B)].

Biomechanical result

The mechanical properties of the tissue-engineered bone from the site reconstructed by *BMP-2* infected MSC cells in Group IV were very close and only slightly inferior to normal cranial bone (81.112 ± 5.433 vs. 86.820 ± 2.793 MPa, respectively, $p = 0.109$).

TABLE II
Areas of New Bone Formation Measured by 3D CT (cm^2)

Group	I		II		III		IV	
Implantation period	6w	3M	6w	3M	6w	3M	6w	3M
Adv-βgal	2.13 ± 0.47	2.87 ± 0.34	2.35 ± 0.33	3.97 ± 0.37	1.01 ± 0.19	2.72 ± 0.43	3.61 ± 0.36	4.41 ± 0.41
Adv-hBMP	3.31 ± 0.33	4.51 ± 0.46	3.17 ± 0.32	2.35 ± 0.19	1.37 ± 0.25	4.17 ± 0.52	4.32 ± 0.50	6.32 ± 0.04*

Values are mean ± standard deviation; $n = 5$ miniature swine/group.
w = week, M = month.
*$p < 0.05$ considered statistically significant.

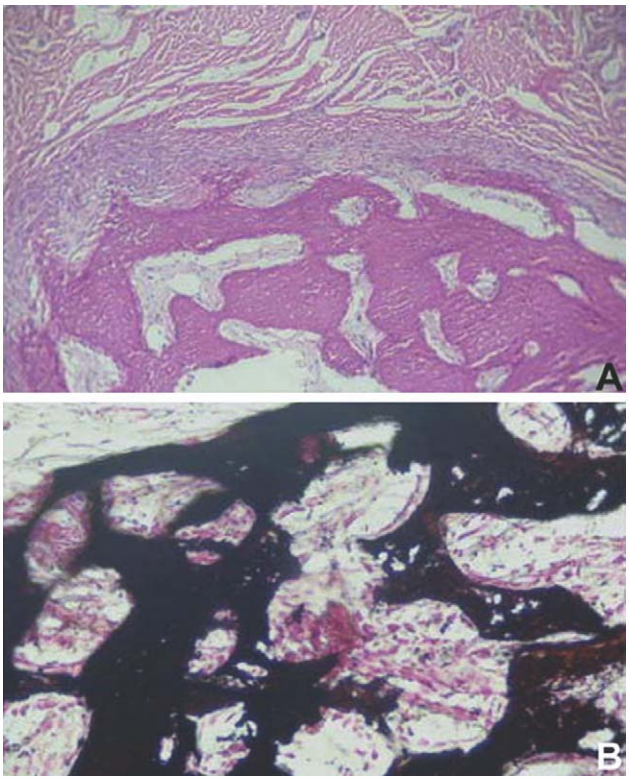

Figure 5. Cancellous bone formation at the cranial defect site implanted with BMP-2 expressing MSC constructs with collagen. (A) H&E staining (×100) (B) Von Kossa staining of the bone specimen (×400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

This study sought to analyze whether the ability of mesenchymal stem cells infected with adenovirus expressing human *BMP-2* to induce bone formation at cranial defects could be enhanced by codelivery with alginate or collagen. Here we demonstrate that the recombinant *BMP-2* containing adenovirus MSC/collagen type I construct could induce bone formation in swine. Importantly, we show definitively that collagen type I scaffold is significantly better than alginate scaffold.

Hydrogels composed of high-molecular-weight alginate exhibit limited biodegradation,[34,35] possibly owing to slow exchange of divalent cation cross-linkers with monovalent cations present in the environment surrounding the hydrogels. There were several methods to reduce the molecular weight of alginate, including radiation,[36,37] acid,[38,39] enzyme treatment,[40–42] and oxidation of alginate.[43] The practice of 8 Mrad or less of Gamma-irradiation treatment favorably causes chain scission within MG-blocks,[44] and was applied for cell transplantation experiments to allow for renal clearance,[45] while still capable of forming stable gels. A constant alginate concentration (2%) was used for the cell transplantation study, to be consistent with earlier studies.[15,46,47]

The degradation rate of scaffolds used for transplanting osteogenic cells noticeably influences following bone formation in the dorsal pockets of SCID mice. Nonirradiated alginate degraded slowly, thus rendered little bony tissue. The irradiated alginate, degraded relatively quickly, allowed more rapid development of bone which was structurally superior to that of more slowly degrading alginate. The mean modulus of the irradiated alginate constructs was only 1.94 ± 0.87 MPa.[48]

Implanting structural polymeric scaffolds mixed with growth factors within RGD-alginate improved the healing of defects. However, functional integration of the constructs was interfered by continued presence of slow-degrading scaffolds and suboptimal dose or delivery of osteoinductive signals.[10] Unlike rat cells, human cells did not readily attach or proliferate on unmodified alginates.[19,49] Chondrocytes in the atelopeptide collagen showed high expression of beta1 integrin, promoting cell-matrix signaling. On the other hand, N-cadherin expression was inhibited in cells mixed with alginate, implying that a reduction in cell-to-cell contact may sustain chondrocyte activity or phenotype.[50]

One study demonstrated that interaction of MSC with the RGD motif significantly inhibited the initial chondrogenesis of MSC within 3D alginate gels.[51] Simmons et al. incorporated BMP-2 and transforming growth factor-beta3 (TGF-beta3) in alginate-RGD, either individually or in combination, and observed significantly more bone formation by the transplanted BMP-2 + TGF-beta3 MSC as early as 6 weeks after implantation compared with individual delivery of BMP-2 or TGF-beta3, which showed negligible bone tissue formation up to 22 weeks.[52] The more rapidly degrading gels led to dramatic increases in the extent and quality of bone formation. These results indicate that biomaterial degradability is a critical design criterion for achieving optimal tissue regeneration with cell transplantation.[48]

Collagen I samples had higher stiffness values than those of alginate at 1, 2, 4, 6 weeks, histologically and radiologically, with ectopic bone formation in the subcutanoues dorsal pockets of nude mice model. All cell-containing samples had higher compression values than acellular ones at 1, 2, 4, 6 weeks, suggesting that the newly formed tissue in cellular specimens contributed to mechanical stability.[14]

Yasko et al. first demonstrated the stimulatory effects of recombinant human BMP-2 protein on bone formation in the rat segmental femoral-defect model.[53] Since then, various studies in the sheep femur[29] and in canine spine and mandible models[28,54–56] have further validated the bone growth enhancing function of BMP-2. Although the

results of direct implantation of BMP into defects are encouraging, a large amount of protein (up to milligram quantities) is often required to stimulate significant new bone formation *in vivo*, and use of large quantities of the protein increases the risk of unwanted side effects such as prolonged edema and ectopic bone formation. Moreover, lack of a system to deliver proteins in a continuous manner over time[57] may hamper its clinical application. Adenovirus-mediated *BMP-2* gene transfer to MSC induced proliferation and differentiation of MSC into an osteoblastic lineage. We as well as many others have speculated that this effect is through an autocrine and/or paracrine mechanism.

Large defects do not regenerate spontaneously during the life span of the animal and are known as critical size defects. We created full thickness 2×5 cm^2 cranial defects bilaterally to ensure that the area would not regenerate within the 3-month observation period. Furthermore, we removed both the dura and periosteum, which have strong osteogenic abilities, to prevent false positive cranial regeneration.[58] Previous work from our laboratory has demonstrated dura to be a potent osteoinductive tissue in animals. The dura was removed in this study to alleviate the concern about possible bone formation from the dura source. Cerebrospinal fluid leakage was prevented by a sheet of acellular dermis (Pig Alloderm, LifeCell).

Transplantation of MSC along with alginate has been previously shown to strengthen osteoporotic bone in rabbits by biomechanical tests. Histomorphometry and histology confirmed more bone apposition in the MSC/alginate-treated group after 8 weeks of implantation.[13] However, size of the defect is a critical factor and larger defects can only be tested in larger animal models. In this study, the larger animal was used to test the biocompatibility and the effectiveness of alginate system in large size defects. Importantly, we found alginate to be inferior to collagen type I, after controlling for all other compounding variables.

Distal femoral articular osteotomies in nude rats have been treated with stem cells transduced with adenoviral *BMP-2* and delivered in alginate carrier. Gene expression at the osteotomy site was confirmed by *in vivo* imaging. In groups treated with stem cell-alginate constructs, bone healing was impeded by the development of a chondroid mass.[10] Our data also revealed the alginate systems hamper both MSC and *BMP-2* gene transfer of MSC.

CONCLUSIONS

Among the four scaffolds tested here, collagen type I (Pancogen) resulted in the best bone formation in the 3-month observation period. Collagen, which degrades more quickly (within 2 weeks), permitted more rapid development of a bony tissue in the cranial defects upon induction by intrinsic BMP-2 released from by *adv-BMP-2* gene engineered MSC. Furthermore, 3D C.T. revealed nearly complete repair of the large size cranial defects by tissue engineered MSC/collagen type I construct. Biomechanical tests demonstrated that the compressive modulus of the new bone was similar to that of the normal trabecular bone (50–100 MPa).[59] Using the same stem cell type and *ex vivo* gene transfer method in this tissue engineered approach to cranial bone regeneration, the hydrogel negatively impacted the success of the constructs.

The use of the MSC/collagen I coupled with *adv-BMP-2* gene transfer enhanced the bone healing in large size cranial defects. These defect sizes are approaching those that are clinically relevant. Taken together, these data demonstrate that appropriate combinations of soluble and regulatory signals in optimal type I collage scaffold in a cell-based tissue engineering systems result in safe, efficient, and effective bone regeneration.

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