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Effect of a Freeze-Dried CMC/PLGA Microsphere Matrix of rhBMP-2 on Bone Healing

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ABSTRACT

The hypothesis of this research was that implants of poly(lactide-co-glycolide) (PLGA) microspheres loaded with bone morphogenetic protein-2 (rhBMP-2) and distributed in a freeze-dried carboxymethylcellulose (CMC) matrix would produce more new bone than would matrix implants of non-protein-loaded microspheres or matrix implants of only CMC. To test this hypothesis it was necessary to fashion microsphere-loaded CMC implants that were simple to insert, fit precisely into a defect, and would not elicit swelling. Microspheres were produced via a water-in-oil-in-water double-emulsion system and were loaded with rhBMP-2 by soaking them in a buffered solution of the protein at a concentration of 5.4 mg protein per gram of PLGA. Following recovery of the loaded microspheres by lyophilization, matrices for implantation were prepared by lyophilizing a suspension of the microspheres in 2% CMC in flat-bottom tissue culture plates. Similar matrices were made with 2% CMC and with 2% CMC containing blank microspheres. A full-thickness calvarial defect model in New Zealand white rabbits was used to assess bone growth. Implants fit the defect well, allowing for direct application. Six weeks postsurgery, defects were collected and processed for undecalcified histology. In vitro, 60% of the loaded rhBMP-2 released from devices or microspheres in 5 to 7 days, with the unembedded microspheres releasing faster than those embedded in CMC. In vivo, the rhBMP-2 microspheres greatly enhanced bone healing, whereas nonloaded PLGA microspheres in the CMC implants had little effect. The results showed that a lyophilized device of rhBMP-2/PLGA microspheres in CMC was an effective implantable protein-delivery system for use in bone repair.

KEYWORDS: bone morphogenetic protein-2, PLGA microspheres, controlled delivery, protein delivery, in vitro, in vivo, bone repair

INTRODUCTION

Until the mid-1980s, research on the newly cloned proteins of pharmaceutical importance was difficult because of the scarcity of the factors for study. Biotechnology changed the situation, and now many factors are in clinical studies. Although the Food and Drug Administration has approved a number of protein drugs, the drugs are usually not effective with oral administration because of low bioavailability. This stems from very poor absorption and enzymatic degradation. Intravenous administration has been used effectively, but the drugs suffer from a very short plasma half-life [1] and frequent administration is necessary. For efficacious use of some proteins, targeted or local delivery is required.

Many systems have been developed to localize growth factors [2-16]. Several controlled-release formulations have been approved (eg, Leutinizing hormone releasing hormone) agonists, tetanus toxoid, human growth hormone [1-3]). Most often, the approach to controlled delivery uses biodegradable or nonbiodegradable polymers as encapsulation agents, either as microspheres or depots.

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a 32-kd homodimeric protein presumed to promote commitment of multipotential stem cells or progenitor cells to osteoblast lineage [4]. Availability via recombinant DNA technology, cloning, protein

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expression, and purification science [5-11] has allowed intensive research efforts toward the use of rhBMP-2 in bone restoration and repair [12-21]. The protein's osteoinductive property of causing mesenchymal differentiation into chondrocytes, with subsequent calcification of the cartilaginous matrix, can be enhanced by prolonging its presence at the site of healing [22]. Clinical use of rhBMP-2 has been hampered by a lack of suitable systems for its delivery. Such systems should be capable of maintaining the protein in situ for sufficient time for it to interact with target cells, release the protein at effective concentrations during bone formation, cause no unnecessary tissue distress, and be resorbed [23]. Among many systems investigated, biodegradable spheres of poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and the copolymer poly(lactide-co-glycolide) (PLGA) have been selected because they are bioerodible but not toxic or tissue reactive. Microspheric forms are convenient because they can be further processed into injections or depots.

PLGA microsphere delivery systems for rhBMP-2 have been previously reported [16,24-26]. In a study of various carriers for rhBMP-2 microspheres, Rodgers et al [26] found that methylcellulose elicited more fibrosis than did carboxymethylcellulose (CMC) and that CMC alone (ie, with no added rhBMP-2 microspheres) showed a mild promotion of bone growth. However, distinct swelling around implant sites was observed during the first few days of treatment. Based on the possibility that CMC may somehow enhance bone growth, the overall aim of this research was to determine if implants fashioned from PLGA microspheres loaded with rhBMP-2 and distributed in a freeze-dried CMC matrix would produce more new bone than matrix implants of non-protein-loaded microspheres or matrix implants of only CMC. To accomplish this aim, sterile microsphere-loaded CMC implants that fit precisely into a rat calvarial defect were developed. The process included loading PLGA microspheres with rhBMP-2 and suspending the loaded microspheres in a solution of CMC for subsequent lyophilization and cutting the dry wafers to fit the defect. A target dose of 90 μg was chosen because it had been tested previously in a rabbit calvarial model [26]. A bilateral 7.9 mm defect size was used because preliminary work had indicated no apparent effect of protein from the treated defect to the untreated defect. The previous study had shown that nontreated defects do not heal or change substantially in a 4- to 8-week period, and based on those results, a 6-week healing period before evaluation was chosen. Creating the control nontreated defect in each animal allowed subtraction of any natural bone healing response inherent in that animal from response as a result of treatment.

MATERIALS AND METHODS

Materials

Lyophilized rhBMP-2 (lot FD74) and 50:50 PLGA porous microspheres (lot 4A19D014) were obtained from Genetics Institute, Inc, Andover, MA. Lyophilized protein was reconstituted with water for injection; the resulting solution was concentrated approximately 10-fold by ultrafiltration on a Diaflo YM 10 membrane (Amicon, Inc, Beverly, MA). Protein solution was aseptically filtered with 0.2 mm filters (Millex GV, Millipore Corp, Bedford, MA) and stored at 4°C. The microspheres had a weight average molecular weight of approximately 32,000 g/mol as determined by gel permeation chromatography [27], a bulk density of 0.18 g/mL, and a specific surface area of 0.58 m2/g. Pharmaceutical grade sodium CMC (type 99-7HF) was obtained from Aqualon Chemical Company (Wilmington, DE). Falcon 12-well, flat-bottom, multiwell tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) were used to lyophilize CMC or microsphere-loaded CMC. Other chemicals and solvents were of reagent grade and were obtained from Fisher Chemicals (Fair Lawn, NJ), Aldrich Chemical Company, Inc (Milwaukee, WI), and Sigma Chemical Company (St Louis, MO).

Determination of optimal microsphere/CMC ratios

CMC solutions of 2.0%, 2.5%, and 3.0% (wt/vol) were prepared according to manufacturer's instructions, then steam sterilized at 121°C, 15 psi for 20 minutes. Microspheres were suspended in the CMC at up to 208 mg/mL, and suspensions were applied to flat-bottom tissue culture plates. Plate wells were filled to approximate bed heights of 0.2 or 0.3 cm. Suspensions were assessed for their ease of mixing and to ensure uniform coverage of the well bottom.

Preparation of rhBMP-2 loaded microspheres

The rhBMP-2/microsphere interaction technique has been described [24,25]. Briefly, the microspheres were

suspended in a protein solution and allowed to equilibrate for 24 hours at room temperature (RT) before recovery by filtration on a 0.45 μm low-protein-binding filter. Wet microspheres were lyophilized via (1) freezing at -45°C for 6 hours, (2) primary drying at 15°C, 150 mTorr for 12 hours, and (3) secondary drying at 25°C, full vacuum, for 6 hours. Subsequent quantification of "free," "bound," and "total" rhBMP-2 associated with the microspheres was carried out by using a simple protein mass balance and the assumption that free protein concentration in the PLGA microspheres was equivalent to that in the separated rhBMP-2 solution following the filtration step. Free protein referred to that present on the surface and within the microspheres' pores of the PLGA matrix, whereas bound protein referred to that physically adsorbed. Loaded microspheres were assessed by scanning electron microscopy and for protein load and in vitro release.

Preparation of lyophilized CMC and microsphere-loaded CMC devices

Flat-bottom tissue culture plates were filled with CMC solutions or microsphere/CMC suspensions and lyophilized by the cycle described previously. Dried wafers were cut into 7.9-mm-diameter disks and stored at 4°C. Wafers for in vivo experiments were weighed. rhBMP-2-containing devices were assessed for protein load and release profile.

Protein quantification and release

Microspheres or devices were assessed for total protein by extraction with a solution of 0.5 M arginine, 0.5 M sodium chloride, 50 mM potassium phosphate monobasic, and pH 7.5 (high salt buffer). Protein concentrations were determined with reverse-phase high-performance liquid chromatography, as previously described [25]. Protein release was determined by incubating the loaded microspheres or devices in isotonic phosphate (50 mM) buffered saline (PBS), pH 7.4 with 0.02% sodium azide at 37°C. At 1, 3, 5, 7, 14, 21, 28, and 35 days, the tubes were centrifuged and the remaining wet material weighed. The collected supernatant was assayed for protein concentration, and PBS was added as replacement for continued incubation.

In vivo evaluation

In these studies both the surgeon performing the implantations and the histologist evaluating specimen slides were blinded to the treatment groups. Animals were randomly selected. Two 7.9-mm-diameter full-thickness calvarial defects were created in 3 groups of 10 rabbits. The left defect was an untreated control, whereas the right defect had 1 of 3 types of implants: (1) CMC, (2) CMC with PLGA microspheres, or (3) CMC with rhBMP-2 loaded microspheres (90 μg rhBMP-2). Previous work [26] had shown that a subjective evaluation (with n = 4 evaluations) performed in a larger diameter defect model at 2, 4, and 8 weeks postoperatively could be effectively used for trend analysis and that peak bone healing may occur between 4 and 8 weeks. A 6-week healing period was thus selected and the number of subjects in each group increased (to n = 10) to increase the probability of seeing significant differences in a subjective evaluation. After 6 weeks, the defects were collected and processed for undecalcified histology as previously described [26]. Slides were evaluated for histologic features considered of importance in bone healing, namely, inflammation (considered negative at the 6-week point), new bone amount (considered positive), new bone type (considered positive depending on the type), collagen (considered positive), fibroblasts, fibrosis, fat (considered negative), how far the edge of any new bone extended (considered positive), and vascularity (considered positive) (Table 1). Each feature was rated on a scale of 0 to 3. Then, for each rabbit, the value obtained from the left (untreated) defect was subtracted

Table 1. Histological Features Considered Important in Bone Healing Used to Evaluate Rabbit Calvarial Implants*

Score:	0	1	2	3
Inflammation, macrophages, giant cells	severe	moderate	Some	none
New bone amount	none	scattered islands	thin sheet	bone table with trabeculae
New bone type	none	woven	Mixed	lamellar
Collagen	none	mild	moderate	effusive
Fibroblasts, fibrosis, fat	large amounts	moderate	Small amounts	none
Defect edge bone	none	some	moderate	extensive, extending into defect
Vascularity	none	few	moderate	many

*Scoring system for each feature is shown in top row.

from the value for the right (implanted) defect. The mean for each factor (and also the combined factors of new bone amount plus type) were analyzed separately across the 3 groups for significant differences. These raw data were analyzed by one-way analysis of variance (ANOVA), with post hoc Tukey-Kramer multiple comparisons testing. National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed.

RESULTS

In vitro

Lyophilized matrices of 2.0%, 2.5%, and 3.0% CMC containing 0 to 208 mg of PLGA microspheres were produced to determine the percent CMC solutions that are readily poured, dried, cut, and handled and to determine the mass of PLGA suspendable in an implantable device without compromising processing or handling ease. All of the lyophilized CMC suspensions were easily cut with sharpened, sterile cork borers or scalpels. Three percent CMC, after autoclaving, was highly viscous, preventing pouring for lyophilization; the solution did not spread out enough on a flat-bottom plate. The 2.5% and 2.0% CMC solutions poured and spread better, respectively. However, adding microspheres to 2.5% CMC increased viscosity and made handling difficult. The limit was 83.3 mg PLGA/mL. With 2.0% CMC, suspensions up to 208 mg PLGA/mL were processable. Two percent CMC was chosen for preparing devices for in vivo experiments. Microspheres (0.5 g) were loaded with rhBMP-2 by soaking in 1.85 mL of solution at 4.4 mg/mL for 24 hours at RT. The microspheres bound 0.38 mg rhBMP-2/g PLGA and retained 7.03 mg rhBMP-2/g PLGA as free protein. This is in contrast to the binding reported by Duggirala et al [24] of 0.54 mg/g PLGA using similar microspheres but a rhBMP-2 concentration of 0.3 mg/mL. It is possible the high protein concentration used in this study favored protein-protein interactions over protein-polymer interactions. Protein load amounts calculated from the binding experiments were confirmed by extraction of rhBMP-2 with high salt buffer. Scanning electron micrographs of microspheres with and without protein are shown in Figure 1. The protein imparts a coating of material (Figure 1b). Higher magnification (Figures 1c, 1d) reveals how the lyophilized protein coat smoothed the surfaces but the microspheres remained spherical and porous (Figure 1b).

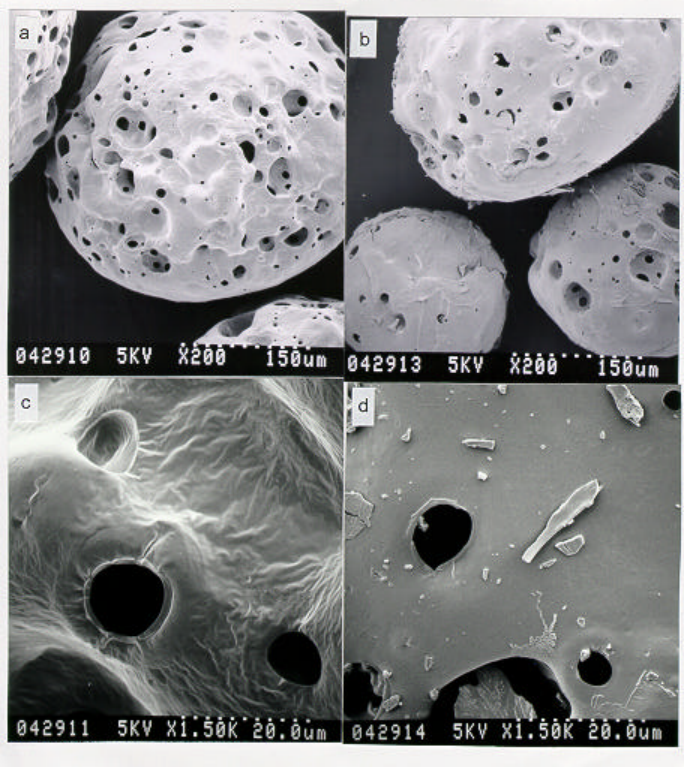

Figure 1. Scanning electron micrographs of blank (a, c) and protein-loaded microspheres (b, d).

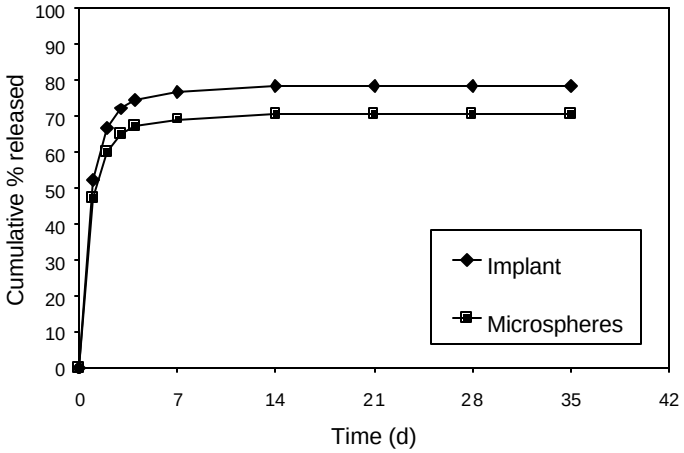

Figure 2. Bone morphogenetic protein-2 release from microspheres and microsphere-loaded carboxymethylcellulose device.

Figure 2 shows rhBMP-2 release from microspheres and the matrix device. The profile shows a burst of protein (= 50%) on the first day with a total of 70% to 80% of the loaded protein released from the microspheres or devices in 5 to 7 days. This corresponds to the early release profiles reported for similar systems [24,26,28]. Duggirala et al had reported that unbound rhBMP-2 releases first, in 5 to 7 days, followed by slow release of bound protein starting at

around 4 weeks. Although expected, no rhBMP-2 release was detected at 4 weeks or beyond for the microspheres in this study. This is due to the relatively small amount of bound rhBMP-2 available in the microspheres used.

In vivo

Table 2 compares the makeup of each treatment device. Figure 3 represents the device insertion process and illustrates its ease of use. Figure 4 shows representative samples of the histology analysis of each treatment. Clearly, no treatment results in little healing, with untreated defects producing only occasional islands of new bone emanating from the dura, a potential source of bone growth factors. Treatment with CMC or microspheres (with no rhBMP-2) in CMC does little to enhance healing. Islands of bone formed along the dura margin, but a full table had not formed by week 6. In contrast, defects treated with the rhBMP-2 microspheres in the device produced 2 bone tables, at the periosteum and the dura margins. The bone appeared to be maturing, tending toward remodeling with lamellar bone. Figure 5 shows the scoring analysis

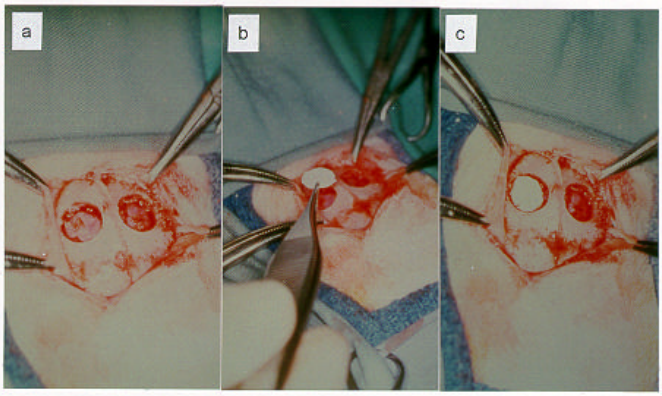

Figure 3. Defects and insertion of devices. a) Two full-thickness 7.9-mm-diameter defects in rabbit calvarium; b) implantable rhBMP-2 delivery device being inserted; c) fully inserted device in 1 defect with opposite side left untreated.

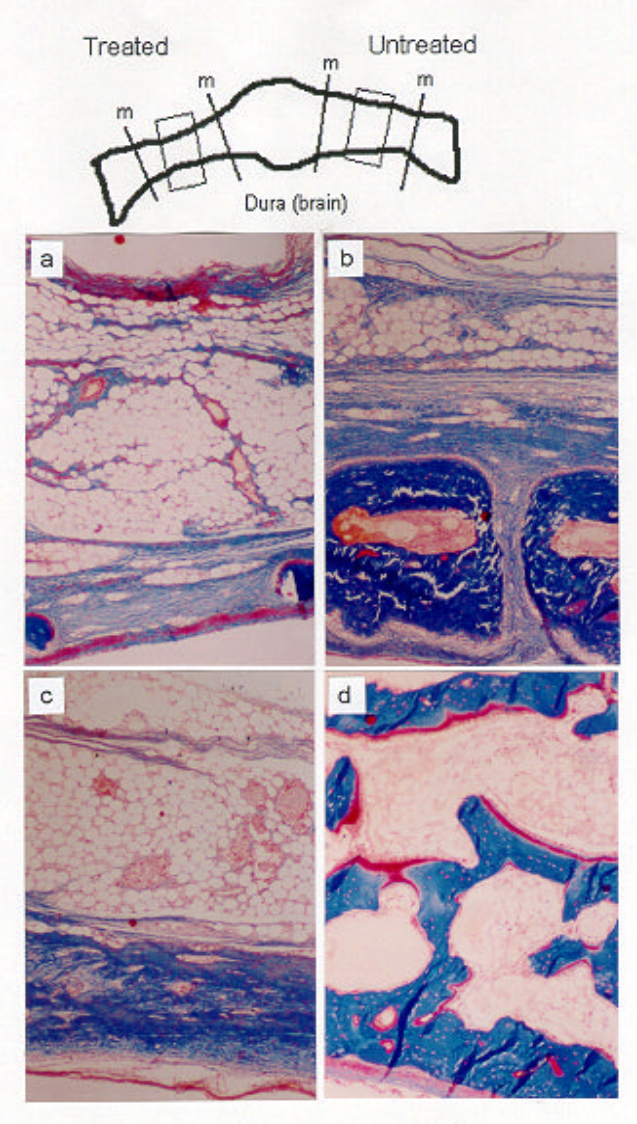

Figure 4. Photomicrographs of cross-sections from rabbit calvarial defects. Masson-Goldner trichome stain magnification ×4. a) Untreated; b) carboxymethylcellulose; c) carboxymethylcellulose and microspheres; d) carboxymethylcellulose, microspheres, and bone morphogenetic protein-2 . The diagram, shown for orientation, represents a 4 μm cross-section of the skull including the treated and untreated defects. The photomicrographs span from the dura to the periosteum. Rectangles show approximate locations for the photomicrographs. m indicates margins of defect.

Table 2. Comparison of 3 Treatments Used in Rabbit Calvarial Defect

Implant	CMC	CMC/Microspheres	CMC/Microspheres/rhBMP-2
Dimensions (height x diameter)	2 mm x 7.9 mm	2 mm x 7.9 mm	2 mm x 7.9 mm
Volume	98 μL	98 μL	98 μL
rhBMP-2 dose	0	0	90 μg
PLGA/implant	0	16.67 mg	16.67 mg
rhBMP-2/PLGA (wt/wt)	0	0	7.42 mg/g
CMC/implant	1.96 mg	1.96 mg	1.96 mg

CMC indicates carboxymethylcellulose; rhBMP-2, bone morphogenetic protein-2; PLGA, poly(lactide-co-glycolide).

of the 3 treatments used in the full-thickness rabbit calvarial defect. The nontreated defect served as a control for each animal. Treatments resulted in inflammation scores no different than those with nontreatment, and there was no difference among treatment groups regarding inflammation. Likewise, the type of treatment had no significant effect on the amount of vascularity or fibrosis, fibroblasts, and fat.

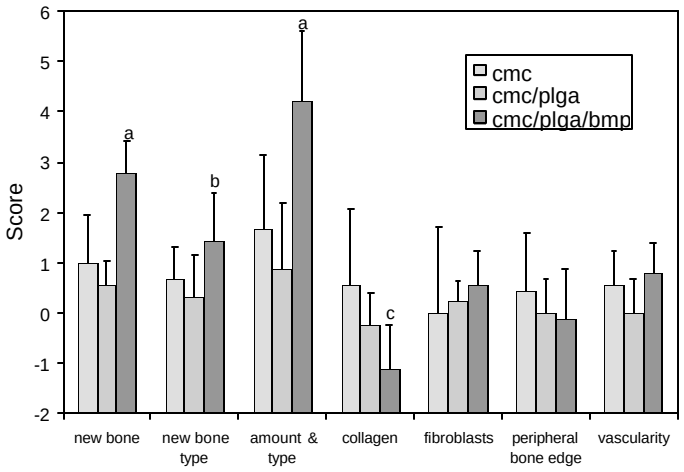

Figure 5. Mean scores of bone growth parameters from histology of in vivo experiments (reported as mean + 1 SD); significance established at *p* = .05. a indicates significantly different vs carboxymethylcellulose (CMC) or CMC/poly(lactide-co-glycolide) (PLGA) groups; b, significantly different vs CMC/PLGA group; c, significantly different vs CMC group. In all cases, Bartlett's test for equal variances established that there were no significant differences in variances between groups (n = 10).

The ANOVA analysis indicated significant differences in several factors analyzed. In comparing the groups, there were no significant differences among any factors between CMC with or without PLGA microspheres. The microspheres neither enhanced CMC's ability to heal bone nor inhibited it. However, when the rhBMP-2/microsphere containing treatment is compared to CMC, there were significantly higher scores for new bone amount, the sum of new bone amount and type, and the amount of collagen present. Compared to CMC/microsphere treatments, the CMC/microsphere/rhBMP-2 treatment scored significantly higher for new bone amount, new bone type, and the sum of new bone amount and type. The histological scores show clearly that rhBMP-2 in a PLGA/CMC delivery device enhanced bone healing in the rabbit calvarial defect.

DISCUSSION

The need to deliver rhBMP-2 to its target cells in optimal timing and dose has been stated repeatedly [28-30]. Not yet available is the knowledge of what is the optimal dose and timing. Such studies have been elusive partly because of a lack of sterile implantable devices that were capable of controlled release. Lyophilized matrices of MC and CMC, containing PLGA microspheres loaded with rhBMP-2, have been suggested and partially tested as 1 such implant type [16-26]. That work was unable to show statistically significant increases in new bone growth because of the presence of rhBMP-2. This was partially the result of a small number of animals being evaluated. One trend noted was a possible enhancement of bone growth by CMC.

The averaged scores of the histological markers showed clearly that CMC alone had no better bone growing capability than it did when it contained PLGA microspheres or rhBMP-2 loaded PLGA microspheres. The implants that contained rhBMP-2 grew significantly more new bone than either type of implant without the BMP. The rhBMP-2 implants also produced more mature bone than did implants not containing the rhBMP-2. Because protein release rates could be altered in the implant by varying the type of PLGA used [31], the system (implant plus animal model) tested should be useful for studying the effects of rhBMP-2 delivery profile on bone growth.

CONCLUSIONS

The results show that a lyophilized device of rhBMP-2/PLGA microsphere suspension in CMC can be an effective implantable protein delivery system for use in bone repair; bone healing was greatly enhanced by the addition of rhBMP-2. The technique used in this study to load microspheres, for adsorption, and for lyophilization is gentle and avoids the protein degradation observed from direct incorporation into PLGA polymer.

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