Enhancement of Bone Growth by Sustained Delivery of Recombinant Human Bone Morphogenetic Protein-2 in a Polymeric Matrix

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Purpose. The purpose of this study was to develop a polymeric sustained delivery system for recombinant human bone morphogenetic protein-2 (BMP-2) and to evaluate local bone growth induced by the sustained release of BMP-2 in an animal model.

Methods. BMP-2 was incorporated in biodegradable poly(D,L-lactide-co-glycolide) (PLGA) microspheres to obtain different release rates. Two sustained and an immediate release implants were produced by suspending the BMP-2 loaded PLGA microspheres in aqueous sodium carboxymethylcellulose (CMC), lyophilizing, and cutting the dried materials to the size of the animal bone defects. The local *in vivo* release at the implantation site in rat calvarial defects was determined by gamma scintigraphy using radiolabeled BMP-2. The local bone induction in the critical size of rabbit calvarial defects was evaluated six weeks post implantation.

Results. The immediate release implant showed about 65% initial drug release within 24 h and the remaining BMP-2 quickly exhausted from the implantation site within 7 days. The sustained release implants, showing 45–55% initial release followed by a prolonged release for 21 days, released a greater amount of BMP-2 at the implantation site and maintained higher serum BMP-2 for the longer period of time compared to the immediate release implant. Significant bone growth was observed in all BMP-2 treated defects while the defects without treatment or with BMP-2-free implant showed minimal bone healing. 75–79% of rabbit calvarial defect area was healed with newly induced bone matrix by the sustained release implants in 6 weeks as compared to 45% recovery from the immediate release implant.

Conclusion. The sustained delivery of BMP-2 based on the biodegradable PLGA microsphere system resulted in faster and more complete bone healing in the animal model.

KEY WORDS: BMP-2; bone healing; PLGA; microsphere; sustained delivery.

INTRODUCTION

The materials available for treating bone defects due to periodontal disease, surgical resectioning, trauma, restoring bony tissue, anchoring dental implants, or repairing and regeneration of dentin and periodontal tissue are limited (1–3). To restore bone defects to normal form and function, surgical implants must be developed to encourage rapid growth.

Through the efforts of biotechnology, a bone growth factor, recombinant human bone morphogenetic protein-2 (BMP-2), has been cloned. BMP-2 induces bone formation *in vivo* (4–10) presumably by stimulating differentiation of mesenchymal stem cells toward an osteoblastic lineage, thereby increasing the number differentiated osteoblasts capable of forming bone (11–12). This stimulative effect on osteoblastic differentiation by rhBMP-2 is of major importance during bone healing in which extensive bone formation capacity from differentiated osteoblasts is needed (13).

BMP-2 has strong bone-inductive activity and is being evaluated as a bone growth inducer for dental and orthopedic indications. Despite its strong osteoinductive activity, clinical use of BMP-2 has been hampered by the lack of suitable delivery systems. Systems evaluated as carriers to localize BMP-2 include porous hydroxyapatite (14,15), absorbable collagen (16–18), polylactic acid (PLA) (19,20), PLGA (21–23), demineralized bone powder and bovine collagen type sponges (24). Hydroxyapatite is a biocompatible but not biodegradable material and therefore remains at the defect site. Collagen sponges can be immunogenic and demineralized bone powder suffers from insufficient supply and poor characterization as a delivery system. Synthetic polymers like PLA and PLGA offer many advantages over biological materials, e.g., biocompatibility, minimal immunogenicity, biodegradability, and the fact they can be manufactured with high reproducibility.

An efficacious delivery system is still needed to localize BMP-2 at the defect site for prolonged periods of time with appropriate dose. Because bone growth is temporal, rhBMP-2 responsive cells may not be attracted to a defect site until after much of the protein has diffused from the site. *In vitro* studies have suggested that a dose response can be produced with appropriate timing of delivery of the growth factor to the cells. The longer the cells were exposed to BMP-2, the more fully they expressed and sustained osteoblastic traits (25,26).

In previous studies, a unique BMP-2 delivery system was developed in which a technique of protein adsorption followed by lyophilization was used to load BMP-2 to biodegradable PLGA microspheres (22,27–29). Total loading and binding of BMP-2 to PLGA microspheres varied by the molecular weight, acid number, and hydrophilicity of PLGA polymers.

In this study, sustained release systems for prolonged BMP-2 release *in vivo* as well as an immediate release system were formulated using individual or combinations of porous microspheres prepared using different PLGA polymers and distributing such in a lyophilized matrix of carboxymethyl cellulose (CMC). The final goal of this research was to address whether sustained release of BMP-2 based on biodegradable PLGA microspheres enhances bone growth more effectively than immediate release within bone defects.

METHODS

Materials

Recombinant human bone morphogenetic protein-2 (BMP-2) and γ-irradiated porous microspheres prepared with

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50:50 poly(D,L-lactide-co-glycolide) (PLGA) RG503 (MW 31 000, hydrophobic end group) were obtained from Genetics Institute, Inc. (Andover, MA). 50:50 PLGA polymers, RG501H (MW 8 100, hydrophilic end group) and RG503H (MW 28 000, hydrophilic end group), were obtained from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA, MW 30,000–70,000) was obtained from Sigma Chemical Company (St. Louis, MO). Pharmaceutical grade sodium carboxymethylcellulose (CMC, type 99-7HF) was obtained form Aqualon Chemical Company (Wilmington, DE). $Na^{125}I$ was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Iodo-Gen® iodination reagent was obtained form Pierce (Rockford, IL). 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).

Protein Determination

BMP-2 concentration was determined by HPLC using a Vydac C-4 reversed-phase column. A gradient elution was performed using 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in 90% acetonitrile (solution B). BMP-2 was eluted by increasing the amount of solution B from 0 to 100% over 13 min at a flow rate of 2 ml/min. BMP-2 was detected at UV 214 nm. The HPLC determination method showed a good linearity without interference in 2-200 μg/ml concentration range ($r^2 \geq 0.999$).

Radio-Iodination of BMP-2

BMP-2 was radiolabeled with ^{125}Iodine using Iodogen® reagent as described in the manufacturer's instruction (Pierce, Rockford, IL). Borosilicate glass tubes (1 × 8 cm) were coated with 100 μg of Iodogen. 0.1 mg BMP-2 in 0.48 ml glycine buffer (2.5% glycine, 0.5% sucrose, 5 mM glutamic acid, 5 mM NaCl, 0.01% Tween-80, pH = 4.5) was then added to the tube. 2 mCi carrier-free $Na^{125}I$ (ca. 20 μl) was added and incubated 25 min at room temperature with gentle shaking. After incubation, the radiolabeled protein was purified on a Sephadex G-25 column (0.8 × 20 cm) using glycine buffer. The first fraction containing the radio-labeled protein was pooled and the specific radioactivity was determined using a gamma counter. The stability and purity of radiolabeled BMP-2 (^{125}I-BMP-2) was assessed by HPLC with the analysis conditions described above. An aliquot of radio-labeled protein fraction was injected on the HPLC column. The column eluent was collected and the specific radioactivity was determined using a gamma counter (Model Cobra II®, Packard Instrument Co. Meriden, CT). The radiolabeled BMP-2 showed a same retention time as intact protein and >99% radioactivity associated with ^{125}I-BMP-2.

Preparation and Characterization of PLGA Microspheres

PLGA microspheres were prepared by a water-in-oil-in-water (w/o/w) double emulsion technique described previously (27). The primary water-in-oil (w/o) dispersion was prepared by introducing 0.5 ml of 1.7 M NaCl in 0.2% PVA solution into 2 grams of PLGA polymer dissolved in methylene chloride and emulsified by sonication for 10 min using a bath sonicator (Branson Ultrasonic Co., Danbury, CT) at room temperature. The dispersion was then introduced into 2 L of an aqueous continuous phase containing 0.2% PVA and 77 mM NaCl. The temperature of the reactor was controlled by circulating water through a double jacketed 2 L glass vessel. Following uniform dispersion, the microspheres were solidified by solvent evaporation/extraction. Solvent removal was accomplished by raising the temperature from 4 to 25°C for 30 min and then to 40°C for 60 min. The microspheres were collected at 25°C by sedimentation, rinsing with deionized water to remove surfactant and freeze-dried. The dried microspheres were sterilized by γ-irradiation at 1.0 Mrad from a ^{60}Co source (Isomedix Corp., Chicago, IL). Surface morphology and porous structures of the microspheres were determined by scanning electron microscopy (SEM) with a Hitachi S-800 SEM (Hitachi, Toktyo, Japan) after palladium/gold coating. Size and size distribution were determined by a Malvern 2600 laser particle sizer (Malvern Instruments, Malvern, England). Specific surface area was determined using a Micromeritics ASAP 2000 surface area analyzer (Micromeritics Instrument Corporation, Norcross, GA). Bulk density was measured by a tapping method.

Preparation of BMP-2 Loaded Microspheres

The preparation was carried out in the GMP facility in the College of Pharmacy, University of Kentucky (Lexington, KY) under aseptic conditions. The sterilized PLGA microspheres were suspended in 0.22 μm filtered BMP-2 (0.5 mg/ml in 2.5% glycine buffer) or ^{125}I-BMP-2 solution (cold : hot = 500 : 1, 0.5 mg cold and 58 μCi hot BMP-2/ml) (g of microsphere : ml of BMP-2 = 1 : 8) and allowed to equilibrate for 24 h at room temperature. The protein loaded microspheres were recovered by filtration using a 0.45 μm filter and lyophilized overnight. Subsequent determination of "free", "bound" and "total" BMP-2 associated with the microspheres was carried out by using simple protein mass balance. "Free" protein, which is able to release immediately from the microspheres, referred to that present on the surface and within the pores of the PLGA matrix, whereas "bound" protein referred to physically adsorbed BMP-2 and whose release from the microspheres follows the degradation of the PLGA matrix. The "free", "bound" protein amounts were calculated from the protein concentrations before and after incubation with microspheres, and "total" protein amounts in the microspheres were assessed by extraction with an extraction buffer (0.5 M arginine and 0.5 M NaCl in 50 mM Na-phosphate monobasic, pH = 7.5).

Preparation of CMC-Based Implants

Three different implants were prepared based on the *in vitro* release profiles of BMP-2 loaded microspheres. An immediate release device was prepared with BMP-2 loaded RG503 microspheres. Two sustained release implants were prepared with two different combinations of BMP-2 microspheres; one with RG501H and RG503H microspheres, and the other with RG501H and RG503 microspheres. Briefly, an aqueous solution of 2.2% CMC was prepared and autoclaved at 121°C, 15 psi for 20 min before use. The BMP-2 loaded PLGA microspheres, 60 mg RG503 microspheres for immediate release formulation; 30 mg RG501H and 30 mg RG503 microspheres for sustained release formulation No. 1; and 30 mg RG501H and 30 mg RG503H microspheres for sustained

release formulation No. 2, were added to 12-Well flat bottom tissue culture plates (Falcon 3043, Becton Dickinson Labware, Lincoln Park, NJ). BMP-2 (cold : hot = 500 : 1, 14.5 μCi/ml, 2 mg/ml in 2.5% glycine buffer, pH 4.5) was added to provide the free BMP-2 required, and 0.4 ml of 2.2% CMC solutions were added to the wells and then mixed with a sterile spatula until the suspension was uniform. Suspensions were lyophilized and the dried wafers were cut into 5.5 mm disks and stored at 4°C. Prepared implants were assessed for protein load and release profile. The implants for the *in vivo* evaluation of bone induction in rabbits were prepared to contain two times greater amount of BMP-2 than the implants for rats, because the size of the rabbit calvarial defect (diameter, 7.9 mm; area, 49.0 mm^2) was two times larger than the rat calvarial defect (diameter, 5.6 mm; area, 24.6 mm^2). The implants were prepared with cold BMP-2 loaded microspheres and cold BMP-2 solution and cut into 7.8 mm disks.

In Vitro Release

A duplicate of 100 mg amount of BMP-2 loaded microspheres or a disc of implant were incubated in 2 ml of 0.1 M phosphate buffered saline (PBS, pH = 7.4) containing 0.02% sodium azide at 37°C. At 1, 2, 7, 14, 21, 28, 35, and 42 days, the sample tubes were centrifuged and the collected supernatant was assayed for the BMP-2 concentration. Fresh buffer was added to replace the supernatant removed.

In Vivo Release of BMP-2 Loaded Implants in Rats

Twelve male Sprague Dawley rats (310–340 g) for each testing group were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Using a 5.6 mm Michele trephine, 5.6 mm diameter full thickness calvarial defects were created. The radioactive BMP-2 implant was placed in the defect and the periosteum and skin were closed. The remaining radioactivity of implants was determined at the time points by γ-radioscintigraphy. A Siemens Basicam® (Siemens, Inc., Chicago, IL) equipped with a pin-hole collimator was set to detect the 35 KeV gamma of ^{125}I and the window was set at 50%. Ten minute static acquisitions were collected using ScinCam® software (GammaForge, Inc., Louisville, KY) on each rat for the following time points 0, 4, 24, and 72 h as well as 7, 14, 21, 28, 35, and 42 days post dosing. Analysis of scintigraphic images was completed using SinWin® software (GammaForge, Inc., Louisville, KY) equipped with a uniform field of view correction software program adapted for pinhole collimators. The collected counts were corrected for the radioactive decay of ^{125}I. Then by using the zero h time point as the original amount of radioactivity administered into the application site, the relative percentage of the amount of radioactivity remaining in the application site for each acquired time point was calculated. Serum BMP-2 levels were determined by collecting serum samples at the time points, precipitating serum proteins with 10% cold trichloroacetic acid (TCA) and counting the radioactivity of the precipitate. This method has been successfully used to characterize rhBMP-2 release from collagen sponges (30).

Evaluation of Bone Growth in Rabbit Calvarial Defect

Fifteen male New Zealand White rabbits for each testing group, age 5–6 months and weighing approximately 4 kg, were quarantined for a period of 1–2 weeks prior to use. Rabbits were anesthetized with an intramuscular or subcutaneous injection of ketamine (5 mg/kg) and acepromazine (55 mg/kg). A #8 bur on the dental drill was used to make an indentation in the defect area for the trephine stylet to rest. Using 7.9 mm Michele trephine, two 7.9 mm diameter full thickness calvarial defects were created. BMP-2 loaded and BMP-2-free (containing blank microspheres) implants were placed in the left and right defects, respectively, and the periosteum was closed with Polysorb (# 4-0) interrupted sutures and the skin was closed with a subcuticular stitches using Dexon (# 4-0). During surgery and 2-week intervals, intravenous fluorochromes were given to mark the newly-growing bone. Oxytetracycline and calcein green, both at 30 mg/kg, were alternated to create lines of different colors in the bone. After 6 weeks, the specimens were collected, fixed in ethanol, and embedded in methylmethacrylate plastic. Decalcified sections (4 μm thickness) were prepared and stained using the modified Masson-Goldner trichrome technique. The sections were evaluated for new calvarial bone area using the Bioquant Elite Bone Morphometry System (R&M Biometrics, Nashville, TN) with a Nikon Labophot microscope (Nikon, Tokyo, Japan) at 40–200× magnification. The values were calculated with Bioquant software and normalized as the percentage recovery of bone tissue (bone + bone marrow) composed of mineralized bone matrix. The data collected from each animal were analyzed with the paired t-tests. *P* value less than 0.05 was considered statistically significant.

RESULTS

Preparation and Characterization of BMP-2 Microspheres

The blank PLGA microspheres, to which BMP-2 can be incorporated by adsorption and lyophilization, were prepared by a w/o/w double emulsion technique. Table I shows the

Table I. Polymer Characteristics and BMP-2 Binding of PLGA Microspheres

	PLGA polymer characteristics			Microsphere characteristics			BMP-2 binding (μg BMP-2/mg microspheres)		
Microsphere	Average MW	End group	Acid number[b]	Bulk density (g/cc)	Average particle size (μm)	Specific surface area (m^2g)	Total	Free	Bound
RG501H	8,000	-COOH	3.1	0.34	83.2	0.192	3.3	0.1	3.2
RG503H	28,000	-COOH	2.3	0.14	134.3	0.916	2.8	0.5	2.3
RG503[a]	31,000	$-CO(CH_2)nCH_3$	0.4	0.17	146.1	0.482	1.6	0.7	0.9

[a] RG503 microspheres were obtained from Genetics Institute, Inc.
[b] Mole of KOH needed to neutralize mole of PLGA.

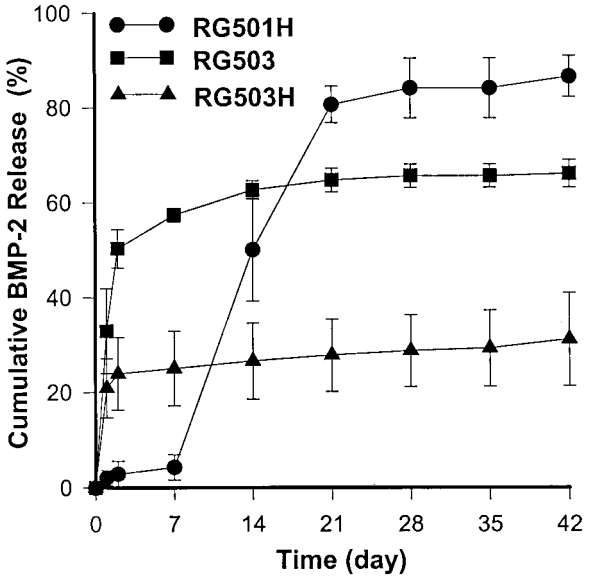

Fig. 1. *In vitro* release of BMP-2 loaded PLGA microspheres in 0.1M PBS (pH 7.4) at 37°C (n = 2).

physical characteristics of the PLGA microspheres. The mean diameters ranged from 83.2 to 146 μm. The bulk densities ranged from 0.14 to 0.34 g/cc and the specific surface areas ranged from 0.19 to 0.92 m^2/g. The blank microspheres prepared with different PLGA polymers incorporated different amounts of BMP-2. The "total" protein loading ranged from 1.6 to 3.3 μg BMP-2 / mg microsphere. Microspheres prepared with PLGA polymers with hydrophilic end group (-COOH), RG501H and RG503H, retained respectively 3.2 and 2.3 μg BMP-2 / mg microsphere as "bound", and 0.1 and 0.5 μg BMP-2 / mg microsphere as "free" protein. RG503 microspheres prepared with a PLGA with hydrophobic end group retained 0.9 μg BMP-2 / mg microsphere as "bound" and 0.7 μg BMP-2 / mg microsphere as "free". A greater amount of "bound" obtained from the greater acid number of the PLGA polymer has been described previously (22). Other physical characteristics (bulk density, particle size and specific surface area) of the PLGA microspheres was not related to the protein incorporation into the microspheres. There was no significant difference in incorporation and binding to the microspheres between the cold and hot BMP-2. Figure 1 shows the *in vitro* release profiles of BMP-2 from the PLGA microspheres. RG501H microspheres showed a very slow release for the first 7 days followed by nearly linear release to 84% between days 8 and 21. The low initial release was due to the low content of "free" protein in the microspheres. RG503H microspheres showed 25% initial release followed by an additional 5% released for 42 days. RG503 microspheres showed over 50% release in first 7 days and an additional 10% in next 7 days. A slow BMP-2 release (< 5%) was observed between days 14 and 42. The initial release of BMP-2 was related to the amount of "free" in the microspheres. The slow release from RG503H and RG503 microspheres, after the initial release of "free", might be due to the slow hydration and degradation of the high molecular weight PLGA polymers.

Preparation and Characterization of BMP-2 Implants

One immediate release and two sustained release BMP-2 implants were prepared with different combinations of the BMP-2 loaded PLGA microspheres (Table II). Microspheres with hydrophobic PLGA (RG503), showing a high initial release followed by a very slow release *in vitro,* were selected to provide a delivery system which would release a large amount of "free" BMP-2 within 7 days followed by maintaining a low drug level at the defect site *in vivo*. The immediate release implant showed about 65% initial release followed by almost no additional drug release for 42 days *in vitro* (Fig. 2). In rats, the immediate release implant (dose = 29.3 μg) released 18 μg BMP-2 within 24 h The amount released from the implant decreased quickly to less than 1 μg between days 7 and 14 (Fig. 3). The immediate release implant and the free BMP-2 in the CMC matrix showed similar *in vivo* release profiles, which suggests that the *in vivo* release pattern of the "free" BMP-2 located in the cavity of the hydrophobic PLGA microspheres is the same as free BMP-2 and not affected by the PLGA polymer matrix. As shown in Fig. 4, the serum BMP-2 concentration with the immediate release implant increased to about 40 ng/ml in 24 h and decreased quickly as similar to the profile of the free BMP-2 implant.

Table II. Preparation and Characterization of BMP-2 Loaded Implants for *in Vivo* Release Kinetics (Rat Study)

		Amount of BMP-2 (μg)					
			Free				
Implants	Microsphere combination	Total	Free in microspheres	rhBMP-2 spiked	Total free	Bound	Percent free
Free BMP-2 in the CMC matrix[a]	—	25.8 (3 μCi)[b]	—	—	25.8	—	100
Immediate release	RG503	29.3 (3.4 μCi)	23.1	—	23.1	6.2	79
Sustained release No. 1	RG501H RG503 (1:1)	35.3 (4.1 μCi)	14.3	8.0	22.3	13.1	63
Sustained release No. 2	RG501H RG503H (1:1)	38.9 (4.5 μCi)	8.5	16.0	24.5	14.4	63

[a] The implant was prepared by mixing cold and hot BMP-2 with 2.2% aqueous CMC, lyophilizing and cutting the dried wafer into the size of the calvarial defect.

[b] The value in the parentheses represents the radioactivity of the implants.

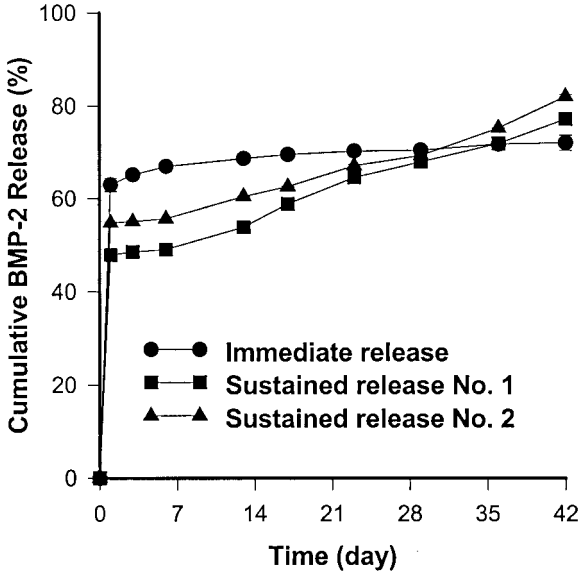

Fig. 2. *In vitro* release of BMP-2 loaded implants in 0.1M PBS (pH 7.4) at 37°C (n = 2).

RG501H microspheres with low molecular weight PLGA were selected and mixed with two different higher molecular weight PLGA microspheres to maintain effective drug level between 3 and 21 days after the initial release of "free" BMP-2. The sustained release formulations showed 45–55% initial releases followed by sustained release to 75–80% for 42 days (Fig. 2). BMP-2 was released from the implant in three phases; a burst release due to the dissociation of the lyophilized "free" fraction, a lag phase when negligible protein delivered, followed by a slow release corresponding to the onset of polymer mass loss and gradual polymer degradation. As shown in Fig. 3, the major difference in the release profiles between immediate and sustained release system is the amount of BMP-2 released between 3 and 21 days *in vivo*. Both immediate and sustained release systems released a similar amount of BMP-2 in the defect site for initial 3 days. After 3 days, the release of BMP-2 from the immediate release system decreased quickly and reached the lowest point at day 21 followed by small release due to the polymer erosion between days 21 and 35. The amount of BMP-2 released from the sustained release system also decreased after 3 days. Compared to the immediate release system, the sustained release system released a greater amount of BMP-2 between 3 and 21 days (Fig. 3). The larger amount of BMP-2 released after 3 days correlated well with the higher serum BMP-2 level between days 4 and 20 (Fig. 4). There was no significant difference in the *in vitro* and *in vivo* release between RG501H/RG503 and RG501H/RG503H combinations of the BMP-2 loaded PLGA microspheres. This suggests that the release of the BMP-2 was mainly controlled by the microspheres with low molecular weight and hydrophilic polymer, RG501H, which showed a sustained release between days 7 and 21 *in vitro*. The two different microspheres with RG503 and RG503H, both showing different BMP-2 adsorption and initial release, seem unable to control the release of BMP-2, except retarding the release of "free" BMP-2 for 2–3 days.

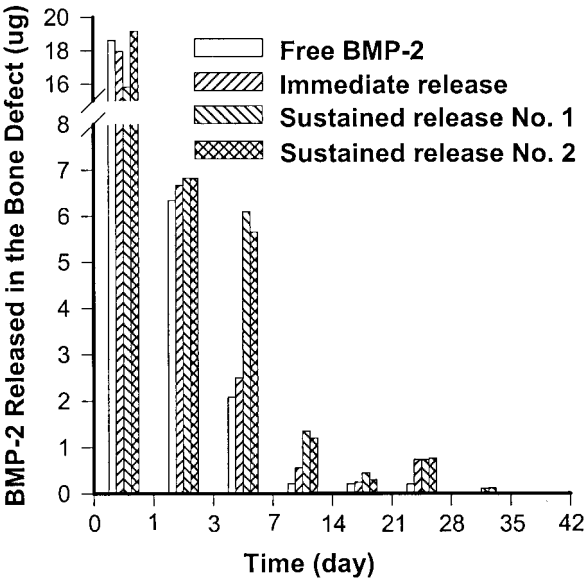

Fig. 3. Amount of BMP-2 released in the rat calvarial defects determined by gamma scintigraphy.

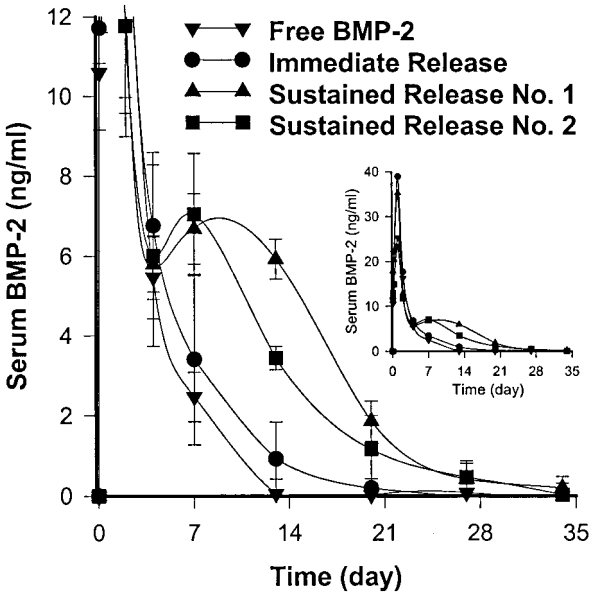

Fig. 4. *In vivo* serum BMP-2 level after implantation of radiolabeled BMP-2 loaded implants in rats.

In Vivo Bone Growth

The control group (no treatment or CMC matrix) showed mainly negligible bone growth and the defects were mainly filled with soft tissues 6 weeks post implantation. Some samples showed minimum bone healing at the edge of the defect (Fig. 5a). The defect treated with immediate release implant showed incomplete and partial bone healing (Fig. 5b). The new bone recovered about 45% of the defect area. The BMP-2 treated defect showed a significant difference ($P < 0.05$) compared to the BMP-2-free control (the implant containing blank PLGA microspheres) as shown in Fig. 6. The sustained release implants induced much higher bone healing compared to the immediate release system. The defects were bridged completely with new bone and the thickness of the new bone was comparable to the original calvarial

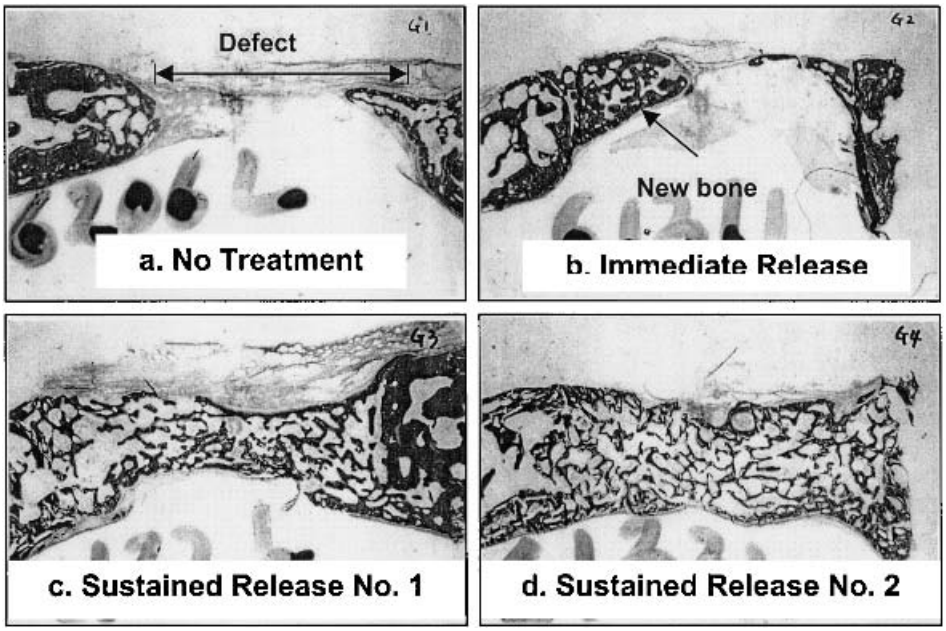

Fig. 5. Microscopic pictures of bone specimens of rabbit calvarial defects six weeks post implantation of BMP-2 implants, a, no treatment; b, immediate release; c, sustained release No. 1; and d, sustained release No. 2.

bone (Fig. 5c and 5d). The new bone occupied 75–79% of the defect area and the sustained release formulations showed a significant difference ($P < 0.05$) with the immediate release for the bone repair at the defect site (Figure 6).

DISCUSSION

The enhancement of bone growth by the immediate and sustained delivery of BMP-2 using a rabbit calvarial defect model was evaluated. The sustained delivery of BMP-2 showed better bone healing than immediate release of BMP-2. The slower release of BMP-2 from the sustained delivery systems maintained higher drug concentration in the defect site for the longer period than the immediate release system in which BMP-2 cleared rapidly from the implantation site. The major difference between the immediate and sustained release systems was the amount of BMP-2 released between 3 and 14 days *in vivo*. Both immediate and sustained release systems released almost same amount of BMP-2 in the defect site for first 3 days. The amount of BMP-2 released in the implantation site decreased quickly from the immediate release system after 7 days. The amount released from immediate release system after 7 days was most likely insufficient to maintain the osteoinductive effects of BMP-2 *in vivo*. The short duration of maintaining effective BMP-2 level is responsible for the incomplete bone healing of the defect. With the sustained release system, the amount of BMP-2 released in the implantation site also decreased after 7 days. However, compared to immediate release system, the sustained release system released a greater amount of BMP-2 than the immediate release system and maintained sufficient amount of BMP-2 in the defect site to stimulate *in vivo* bone healing for up to 21 days. The sustained release system was formulated to contain about 63% free and 37% bound BMP-2 in the implants. Theoretically, the formulation was designed to release 63% free BMP-2 within 3–7 days (9–21%/day = 3 – 7 μg/day) to obtain a high BMP-2 level to initiate bone healing and then release the remaining 37% bound BMP-2 for 3–4 weeks (1.3–1.8%/day = 0.4 – 0.6 μg/day) to maintain the osteoinductive effect. However, the results from *in vivo* release of sustained release implants showed that about 75% of total BMP-2 in the implant released in 7 days (10.7%/day = 3.5 μg/day) and then 17% BMP-2 released for next 14 days (1.2%/day = 0.4 μg/day). This suggests that a continuous release of BMP-2 (>0.4 μg/day) in that particular size of bone defect (24.6 mm^2) maintained by the sustained release system was sufficient to induce osteoblastic responses. The higher concentration and longer release duration of BMP-2 resulted in faster and more complete bone healing in the animal model.

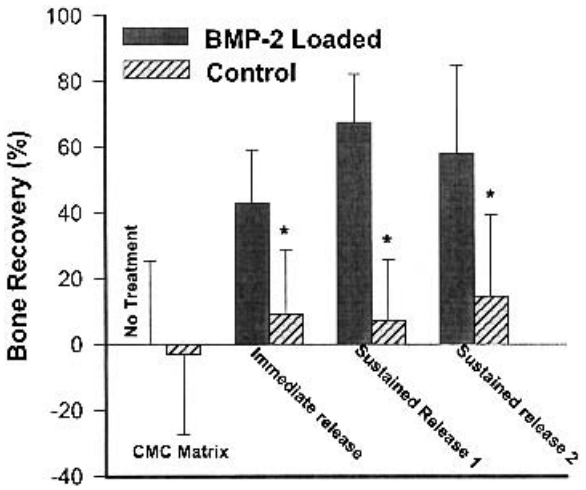

Fig. 6. *In vivo* bone healing of BMP-2 loaded implants evaluated with new bone area. *The calvarial defects treated with BMP-2-free implants containing blank PLGA microspheres.

CONCLUSION

The sustained release system for BMP-2 has been successfully developed based on PLGA microspheres. The sustained release of BMP-2 showed a faster and more complete bone repair than immediate release in the rabbit calvarial defect model.

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