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A new recombinant human bone morphogenetic protein-2 carrier for bone regeneration

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Abstract

A gelatin sponge was formed by foaming and heat treating a gelatin solution, followed by coating the solid with poly(D,L-lactic-co-glycolic acid) to reinforce the gelatin framework. This sponge was tested for its suitability as a biodegradable porous, recombinant human bone morphogenetic protein (rhBMP)-2 carrier. Incorporation of rhBMP-2 into the sponge was closely related to its bulk density of gelatin sponge. The calcium content in the sponges, as assessed by an ectopic bone formation assay in rats, increased with the increasing sponge bulk density. Histologic and peripheral quantitative computed tomography analysis of implants in this ectopic assay system revealed cell growth throughout the carrier in 4 weeks after implantation regardless gelatin bulk density. The carrier containing rhBMP-2 maintained its three-dimensional structure after implantation; the carrier resisted collapse caused by soft tissue pressure during rapid bone formation as assessed by soft X-ray photographs. These results indicate that this newly developed sponge has excellent carrier characteristics to introduce rhBMP-2 into areas needed for bone regeneration.

Keywords: Gelatin; Bone morphogenetic protein; Porous structure; Biodegradable polymer

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1. Introduction

Recombinant human bone morphogenetic protein 2 (rhBMP-2) was cloned by Wozney and coworkers in 1988 (Wozney et al., 1988). Subsequently, rhBMP-2 was shown to induce mesenchymal cells in subcutaneous or muscle tissue to differentiate into chondroblasts or osteoblasts (Wang et al., 1993); in turn, these cells led to the formation of ectopic cartilage or bone (Wang et al., 1990). Consequently, rhBMP-2 combined with an appropriate delivery system has potential in the clinical reconstruction of bone. However, although many potential carriers for bone morphogenetic protein (BMP) have been evaluated, no successful candidate has emerged for clinical use because of the wide range of required characteristics that a successful carrier must possess. One such characteristic of any potential carrier is its ability to be readily replaced and resorbed during bone formation.

Gelatin, made by the partial hydrolysis of collagen, is a frequently used pharmaceutical excipient because it is biodegradable and has low immunogenicity. Aqueous gelatin solutions easily produce a foam that can be stabilized and fabricated as a hemostatic gelatin sponge. The gelatin sponge has a porous structure; the pore size of the sponges can be controlled during the fabrication process. Porous structures are reported to have good characteristics for cell ingrowth and proliferation as previously described (Mikos et al., 1993); therefore, gelatin sponges are excellent candidates for osteoblast scaffolds. Additionally, as rhBMPs are known to have an affinity for collagens (Paralkar et al., 1990), rhBMPs would also have the property for gelatin. Consequently, gelatin sponges would function as an rhBMP reservoir, resulting in the controlled release of rhBMP as degradation occurs in vivo, further stimulating bone differentiation. Although gelatin sponges possess many desirable properties, they lose physical strength and collapse when wetted with body fluids such as blood. This physical weakness is also reported to be a disadvantage of collagen sponges (Lindholm and Gao, 1993).

In order to overcome the physical weakness of gelatin sponges, a new type of carrier composed of gelatin and poly(D,L-lactic-co-glycolic acid) [PLGA] was evaluated. PLGA is a biodegradable and biocompatible polymer that is produced from a mixture of D,L-lactide and glycolide by copolymerization, and it has been widely used for many clinical applications in medical devices, and is generally recognized as safe. This study evaluated the suitability of a new type of biodegradable carrier made of gelatin reinforced with PLGA for the delivery and controlled release of rhBMP-2.

2. Materials and methods

rhBMP-2 was manufactured by Genetics Institute Inc. (Massachusetts) using a Chinese hamster ovary cell expression system. Purified (98%) protein was dissolved in a glycine–glutamic acid buffer. Poly(D,L-lactide-co-glycolide) (inherent viscosity; 0.41 dl/g) was purchased from Boehringer Ingelheim, KG (Ingelheim, Germany). Tween 80 and dioxane were purchased from Kanto Chemical Co. (Tokyo, Japan). Pharmaceutical grade gelatin (JP) was purchased from Miyagi Chemical Co. (Sendai, Japan) and Nitta Gelatin Co. (Osaka, Japan). Calcium standard solution was purchased from Merck KGaA (Darmstadt, Germany). The Iodo-Gen reagent was purchased from Pierce (IL). All other reagents are analytical grade chemicals.

2.1. Fabrication of implant

For each sponge, 4–8 g of gelatin were dissolved in 100 ml of water for injection at 50 °C. This solution was agitated to produce a foam; the foam was cooled gradually to form a gel. Each gel sponge was freeze-dried and dry-heated at 155 °C for cross-link the gelatin. The gelatin sponges were immersed in 8% PLGA and 0.5% Tween 80 dissolved in dioxane, then the solution containing the sponge was lyophilized to remove the dioxane. Heat treatment at 135 °C in dry air was conducted to remove residual dioxane and to coat the surface of each pore with the polymer. The resulting products were designated polymer-coated gelatin sponge (PGS).

2.2. Scanning electron microscope

Sponge samples were coated with gold using a Sputter Coater (JEC-550 Twin Coater;Japan Electron Optics Laboratories [JEOL]; Tokyo, Japan) in a vacuum of 4 Pa and using a coating time 180 s. A JSM-5400 scanning electron microscope (JEOL) was used for observation at a 15 kV beam voltage.

2.3. Light microscopy

Sponge samples with a thickness of about 1 mm were immersed in 0.05% of Amido Black solution (which stains only the gelatin component of PGS) for several minutes at room temperature before observation with a digital microscope (VH-6300, Keyence; Osaka, Japan).

2.4. Rat ectopic implantation assay

The assay was conducted according to the methods described by Reddi and Huggins (1972). Male Fischer rats, 4-weeks-old were used. Sterile incisions (1 cm) were made approximately at the midline of the skin over the thoratic region, and subcutaneous pockets were prepared by blunt dissection at bilateral sites located over the thorax to create sufficient space for implantation of sponge samples. The test samples were prepared by adding 256 μl of rhBMP-2 solution (0.5 mg/ml) or the control solution (a glycine–glutamic acid solution) to $8 \times 8 \times 5$ mm^3 pieces of sterile PGS, followed by incubation for 30 min at room temperature. Two test samples were implanted deep into the pockets, and the incision was closed with a surgical clip. The day of implants was designated as Day 0 of the experiment. Implants were removed on Days 14 and 28 for analysis. Extent of bone formation was evaluated by calcium content, and by histologic and radiographic observation.

Before decalcification, the morphology and radiopacity of the implants were also assessed by soft X-ray photography. Peripheral quantitative computed tomography (pQCT; XCT0960A; Stratec, Germany) was used to determine for calcium distribution within an implant.

The calcium content of the implants was measured by atomic absorption spectroscopy. Briefly after removal, the implants were immersed in 2 ml of 2 N HCl for more than 48 h at room temperature, and 200 μl of the supernatant was analyzed by atomic absorption spectroscopy (SpectrAA-300, Varian Techtron, Australia). The calcium content of each implant was determined by comparing the results against the standard curve obtained by analyzing a standard calcium hydrochloride solution (5, 10, and 20 μg/ml).

After removal, histologic examination was performed on implants fixed in phosphate buffered 10% formalin, decalcified with Na_2EDTA embedded in paraffin, sectioned to a thickness of 5 μm, and stained with azan stain. Specimens were observed with a light microscope.

2.5. Incorporation of rhBMP-2 into PGS

2.5.1. Radioiodination and preparation of rhBMP-2 solutions

^{125}I-labelling of rhBMP-2 was performed with the Iodo-gen reagent (1,3,4,6-tetrachloro-3α, 6α-diphenyl glycouril). Briefly, 50 μl of rhBMP-2 solution (30 μg) and 10 μl of ^{125}I solution were added to a polypropylene centrifuge tube previously coated with the Iodo-Gen reagent. The mixture was incubated for 15 min at room temperature with gentle shaking. The labeled protein was purified on a PD-10 column (Pharmacia; NJ) pre-equilibrated with a glycine–glutamic acid buffer. The resulting ^{125}I-labelled rhBMP-2 solution was diluted with sufficient cold rhBMP-2 solution to make rhBMP-2 solutions of 0.12–3.6 mg/ml.

2.5.2. Measurement of rhBMP-2 incorporated into PGS

Eight hundred micro liters of ^{125}I-rhBMP-2 solution were added to an $8 \times 8 \times 5$ mm^3 piece of PGS. The solution and PGS were incubated together for 30 min at room temperature, and centrifuged at 1500 rpm (800) for 15 min to remove un-incorporated ^{125}I-rhBMP-2. The amount of radioactivity incorporated into PGS was measured using a gamma counter (Aloka, Tokyo, Japan) and the amount of incorporated ^{125}I-rhBMP-2 in

PGS was calculated from the total radioactivity in ^{125}I-rhBMP-2 solution of 0.12–3.6 mg/ml.

2.6. Statistical analysis

Statistical analysis was performed by the use of ANOVA, supplemented with Dunnet's test for multiple comparisons ($P < 0.05$ was considered as significant).

3. Results

3.1. Morphology of PGS

Scanning electron microscopy showed that each PGS was microporous. The size distribution of the pores was heterogeneous, ranging from 50 to 500 μm; each pore was interconnected (Fig. 1). To obtain the exact average pore size is difficult due to the heterogeneity, but pores with the sizes of 200–300 μm were frequently observed by visual inspection. Light microscopy (Fig. 2) revealed every pore of the sponge is fully or partially coated with PLGA, since gelatin is stained by Amido Black. It is assumed that upon heating, PLGA melts and evenly coats the surface of each pore, resulting in a coated structure that reinforces the original gelatin sponge structure.

3.2. Incorporation of rhBMP-2 into PGS

At 0.12 mg/ml, almost all the applied rhBMP-2 was retained by PGSs with the lowest gelatin sponge bulk density (14 mg/cm^3) as well as by PGSs with the highest gelatin sponge bulk density (30 mg/cm^3; Fig. 3). The amount of incorporated rhBMP-2 into PGS increased with the increasing the rhBMP-2 concentration; rhBMP-2 retained by the PGS also increased with increasing gelatin sponge bulk density.

3.3. Rat ectopic bone formation assay

This assay has been used by other researchers to assess the capability of other factors to induce bone formation independent of the nearby presence of living bone (Reddi and Huggins, 1972). In

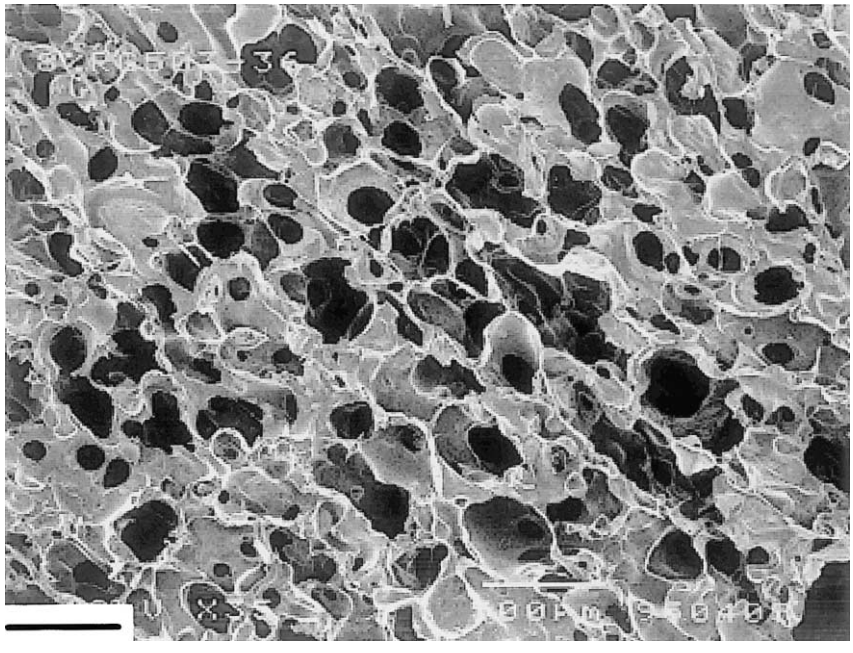

Fig. 1. SEM micrograph of dissected PGS before implantation. The bulk density of the gelatin sponge is 18 mg/cm^3. The bar corresponds to 500 μm.

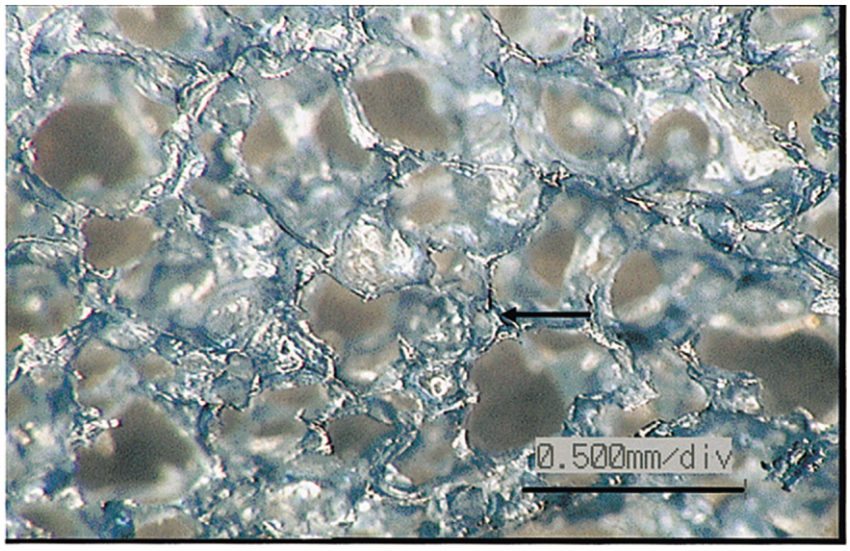

Fig. 2. Light micrograph of dissected PGS after staining by Amido Black. The bulk density of the gelatin sponge is 18 mg/cm^3. The bar corresponds to 500 μm. The arrow indicates the gelatin sponge stained by Amido Black. The gelatin is fully or partially coated with transparent PLGA that is not stained by Amido Black.

the X-ray photographs (Fig. 4), the radiopaque areas were limited to within and only the immediate vicinity of high density PGSs. The intensity of the radiopaque area scarcely increases with increasing gelatin density even if the rhBMP-2 concentration was the same for carriers of different densities. Without rhBMP-2, PGSs did not retain their original shapes, especially having flattened on Day 14 and having shrank on Day 28.

No significant difference in calcium content was observed between PGSs of different bulk densities carrying the same amount of rhBMP-2 on Day 14, however, a significant increase in the bulk density dependency was observed on Day 28 (Fig. 5). PGS without rhBMP-2 did not show any calcium accumulation.

In the pQCT images (Fig. 6), calcification was essentially complete by Day 28. Additionally, the area calcified increased with increasing gelatin sponge bulk density.

Histologic examination (Fig. 7) revealed that on Day 14, bone induction (star symbol) and residual gelatin (small closed arrow) were observed in all implants regardless of gelatin sponge bulk density. Slight bone outgrowth from the implant and cyst (large open arrow) was observed for implants of the lower gelatin bulk density. On Day 28, the calcified areas of implants with the middle and highest gelatin sponge bulk density were obvious and trabecular bone connectivity is also apparent. In contrast, the lower density implants showed low bone volume with poor trabecular connectivity. However, qualitative differences in bone matrix, neo-vascularization and bone marrow formation were not clear among these implants. Infiltration of cells was independent of gelatin sponge bulk density. The increase of the calcified area and trabecular bone connectivity observed by histologic findings correlate well with the pQCT images which show the calcium distribution of the implants by the color. While the remaining amount of gelatin sponge framework (small closed arrows) depends on the initial gelatin sponge bulk density, remnant gelatin was observed in all implants on Day 28 regardless of gelatin sponge bulk density.

4. Discussion

It has been reported that the biological half-life of rhBMP-2 in the blood stream is very short

(Uchida et al., 1996), and that local injection of only a small amount of rhBMP-2 does not induce significant bone formation at the injected site. Consequently, a carrier is needed that sequesters rhBMP-2 at a reconstruction site for the time period clinically demanded for bone to form.

Inactive demineralized bone matrix has been shown to be one such effective substrate (Gerhart et al., 1993; Katz et al., 1993). However, it has many disadvantages for clinical application including: limited availability, sterility assurance, and potential pathogen transmission. Consequently, many other substances, such as hydroxyapatite, tricalcium phosphate (Ohgushi et al., 1990; Wu et al., 1992), PLGA particles prepared from PLGA and whole blood (Smith et al., 1995), fibrin (Kawamura and Urist, 1988), collagen (Kusumoto et al., 1995), polylactide–polyethylene glycol block copolymer (Miyamoto et al., 1993) have been investigated as BMP carriers. However, each of these carriers has its own disadvantage. Hydroxyapatite has sufficient rigidness when implanted, however, it does not degrade for a long-period of time and is not replaced with newly formed bone, resulting in incomplete bone reconstruction. Tricalcium phosphate and PLGA particles have difficulty regulating the shape of the newly formed bone resulting in mineralization in tissues outside the implant. Although collagen is reported to be a good scaffold for cell proliferation (Paralkar et al., 1990), it does not have appropriate physical strength and collapses when implanted into soft tissue (Lindholm and Gao, 1993). Therefore, a successful rhBMP-2 carriers needs: (1) to retain BMP at a bone reconstruction site (controlled release) for a period of time sufficient to induce bone formation; (2) to possess a porous structure to allow cellular infiltration and proliferation, and vascular invasion; (3) to maintain shape against pressures from soft tissue; (4)

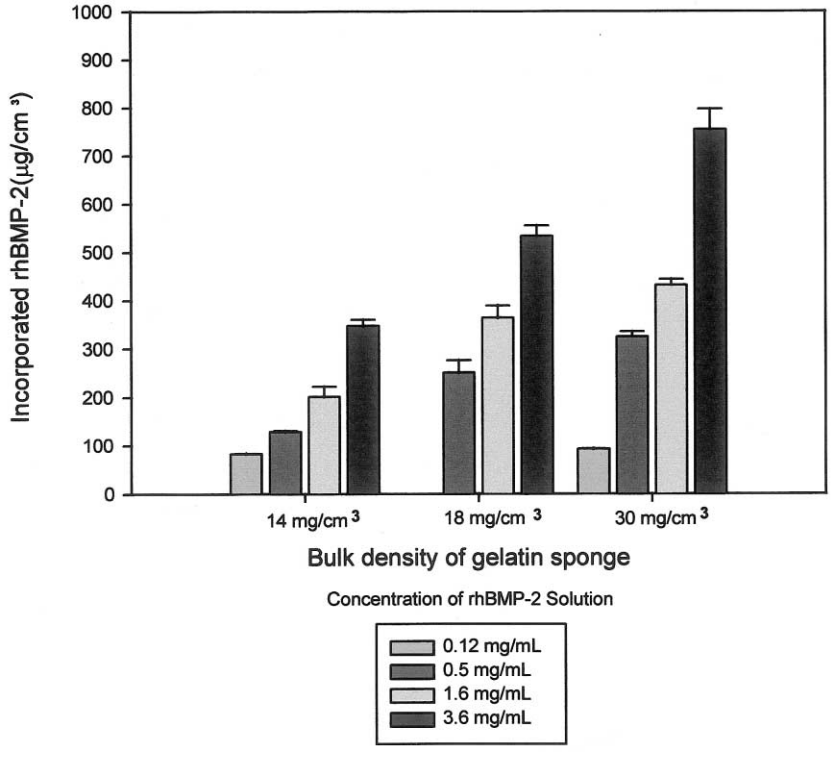

Fig. 3. Incorporation of rhBMP-2 into PGS supports with different gelatin bulk densities. Data for 0.12 mg/ml of rhBMP-2 solution for 18 mg/cm^3 of the bulk density of the gelatin sponge were not obtained.

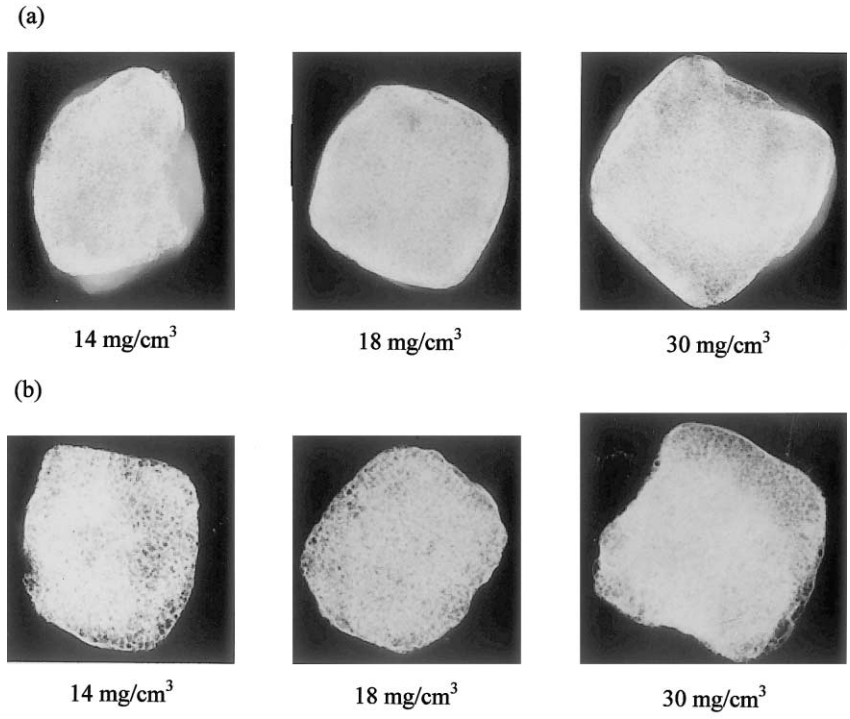

Fig. 4. Soft X-ray photographs of PGS carrying rhBMP-2 and implanted into rats; (a) 14 days after implantation, and (b) 28 days after implantation.

to have low or no toxicity; (5) to be inert, not to interfere BMP activity; (6) to be replaced easily with new bone; and (7) to possess appropriate mechanical strength so that it can be cut or molded into various shapes to fit bone reconstruction sites. Taking these factors into consideration, a new carrier was developed that consists of a gelatin sponge coated with the synthetic polymer (PLGA). This carrier was assessed for its ability to overcome these disadvantages. Of special importance was the ability of the carrier to retain sufficient rhBMP-2 to enhance bone formation. Especially, we focus on the extent of rhBMP-2 retention of a carrier to enhance the ability of bone formation.

Gelatin sponges, which are the basic framework of PGSs, were fabricated by agitating gelatin solutions to a foam, followed by lyophilization to form the structure, and heat treatment to cross-link the gelatin. Although the degree of cross-linking by heat treatment controls the biodegradability of gelatin sponge in vivo, this treatment cannot endow the sponge with sufficient physical strength to resist collapse caused by the surrounding soft tissue. In order to ensure physical strength, PLGA was coated onto the surface of gelatin sponge. The heat treatment following the coating not only removed residual solvent, but coated the PLGA homogeneously onto the surface of each gelatin sponge pore.

It has been reported that the optimal average pore size of a carrier for bone regeneration is 200–400 μm (Ohgushi et al., 1990). Light microscopy revealed that PGSs had the appropriate microarchitecture to achieve vascular invasion, cell ingrowth and osteoconduction in terms of pore size and degree of interconnection. This pore size is defined by the pore size of the gelatin sponge after cross-linking. The PLGA polymer, which is not stained with Amido Black, coates uniformly onto the porous layer of the gelatin sponge (Fig. 2). This PLGA coating by PLGA endows the PGS with physical strength, as shown by the results of rat ectopic bone formation assays.

rhBMP-2 will readily adsorb to collagen as shown by Paralkar et al. (1990) and BMPs were reported difficult to isolate because of their tight adsorption to demineralized bone matrix (Lindholm and Gao, 1993). Gelatin is a hydrolysate of denatured collagen but some part of the three-dimensional structure of collagen molecule is retained. So gelatin also likely has an affinity to rhBMP-2. In the present study as shown in Fig. 3, gelatin sponges manufactured by cross-linking with heat treatment followed by coating with PLGA demonstrated good affinity for rhBMP-2. The binding capacity of gelatin to rhBMP-2 molecule would be saturated above the certain concentration of rhBMP-2. In fact, at the concentration of 0.12 mg/ml, the same amount of rhBMP-2 was retained in PGSs with the bulk density of gelatin sponge of 14 and 30 mg/cm^3, suggesting that, up to this concentration, stoichiometric binding of rhBMP-2 with gelatin molecule would be achieved. Beyond around this concentration, contribution of the hydrogel characteristics of gelatin sponge in the PGS would be greater. In addition to the bound rhBMP-2 with gelatin molecule, the unbound rhBMP-2 can reside within gelatin sponge hydrogel as a free molecule even after extensive centrifugation. Therefore, the amount of rhBMP-2 incorporated into PGS was shown to increase with increasing the concentration of rhBMP-2 in this study. These results indicate the two step release of rhBMP-2 from PGS; the first step is the quick release of rhBMP-2 which resides in the pores of PGS and the second step is the gradual release of the incorporated rhBMP-2 into the gelatin sponge that is coated with PLGA. Due to the presence of PLGA, the release of rhBMP-2 from the gelatin sponge would be relatively delayed when the body fluid comes into PGS. Especially, this effect will be significant at the implant site where bleeding may occur. The first burst like release may contribute to the chemotaxis of osteoprogenitor cells and then the gradual release would enhance the bone induction by rhBMP-2.

Limiting bone formation area to the surrounding of the implant is a significant issue in the development of a rhBMP-2 delivery system in order to reconstruct the bone in as an original

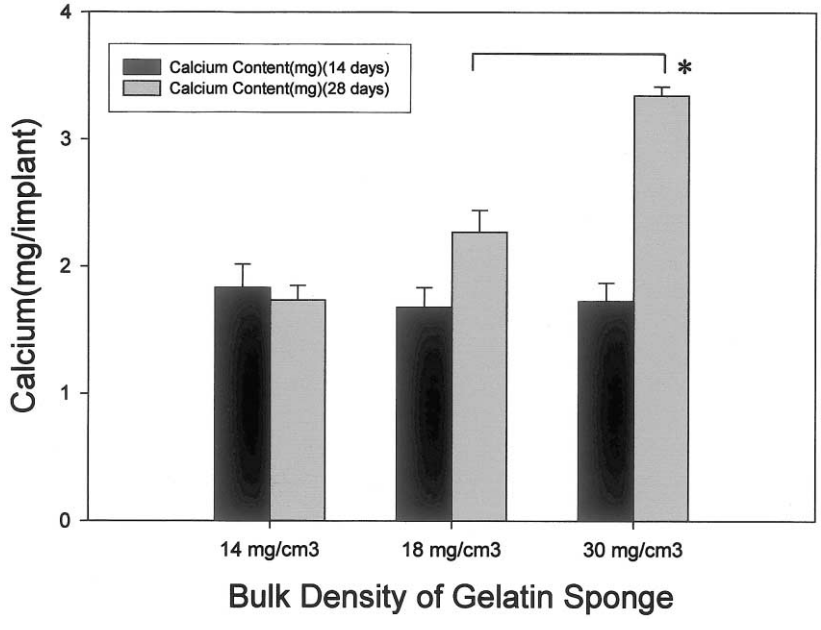

Fig. 5. Calcium content of implants. The amount of added rhBMP-2 was 0.4 mg/cm^3 PGS. Each measurement is the mean ± SEM of ten implants harvested from five rats on 14 days or 28 days after implantation. No calcium accumulation was observed in PGS without rhBMP-2; $P < 0.05$ (Dunnet).

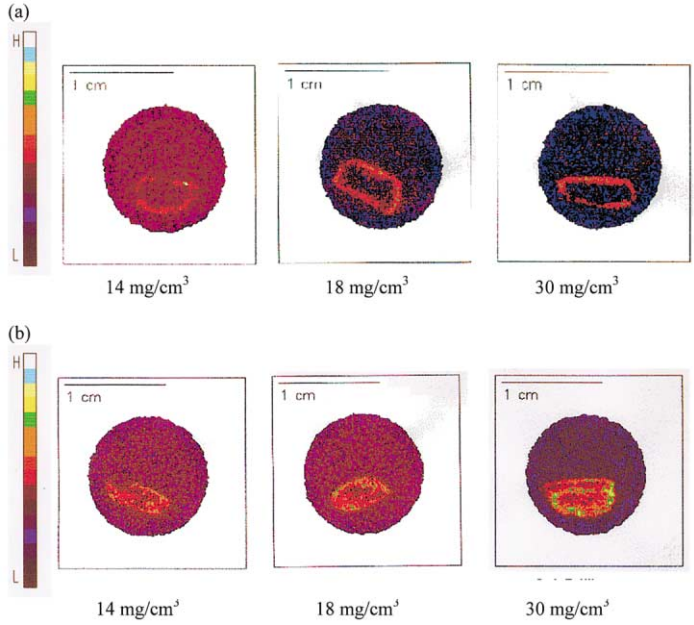

Fig. 6. pQCT images of vertical cross-section of PGS with rhBMP-2 implanted into the rat bilateral pocket for (a) 14 days (or) (b) 28 days.

shape as possible and to avoid any side effects associated with malformed bone. Radiographic examination of implants with soft X-rays after removal has revealed that PGS contains bone formed almost within the implant. The thickness of the implants after implantation slightly reduced from the original one. By Day 28 after implantation, the entire volume of the implants was filled with induced bone, as shown by X-ray and pQCT imaging and by histologic findings, indicating that PGSs carrying rhBMP-2 promote bone formation even at the center of the implants after only 28 days, regardless of gelatin bulk density.

In contrast, control animals implanted with PGSs not treated with rhBMP-2 did not show any evidence of bone formation. Moreover, the PGSs did not retain their original thickness and flattened, obviously due to external pressure. Additionally, the implants were resorbed, their original size had become extensively reduced by Day 28. These results suggest that bone is formed in gelatin sponges only by the presence of active rhBMP-2. Additionally, although cross-linked gelatin sponges alone treated with rhBMP-2 induced bone formation, the calcified tissue was very thin, as was previously reported for collagen sponges (Lindholm and Gao, 1993). This finding indicates that reinforcement of the framework is required to maximize the biological activities of rhBMP-2. The result also raises the question whether gelatin is a necessary component in this system. Indeed, Ishaug showed that stabilized PLGA foams are good scaffolds for rat marrow stromal osteoblast cultures. However, the primary limitation of this culture system is the poor penetration of growing cells to the center of the PLGA scaffolds, leading little bone development at the center of the implant (Ishaug-Riley et al., 1997a,b). Bone tissues were only found at the periphery of several devices incorporated with rhBMP-2 after 14 days but bone formation was observed throughout the PGSs regardless of gelatin sponge density after 28 days. These findings suggest that the efficient growth of cells throughout the PGS is achieved by retaining rhBMP-2 within the gelatin matrix. In addition, bone formation quality and mineral density at a given dose of rhBMP-2 depend on the bulk den-

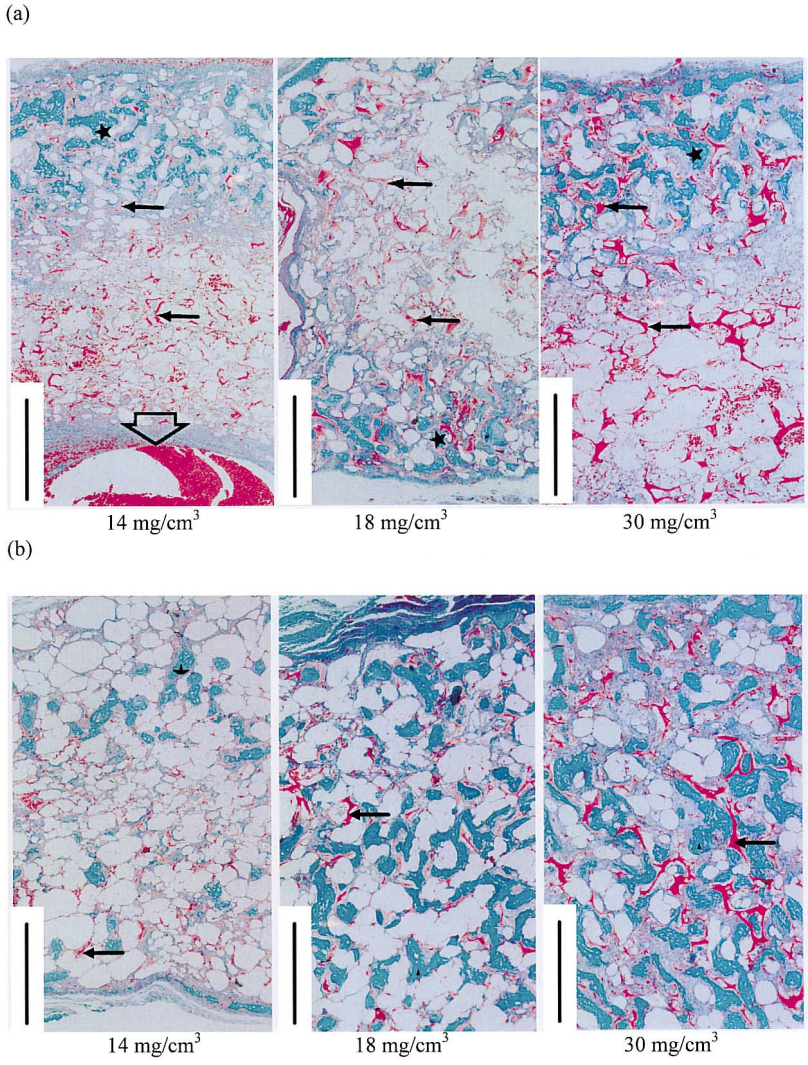

Fig. 7. Histological sections of PGS with rhBMP-2 implanted into the rat bilateral pocket for (a) 14 days, or (b) 28 days. Bar correspond to 500 μm. The large open arrow indicates the cyst formation on the surface of the implant, the small closed arrows indicate remaining gelatin sponge, the stars indicate induced bone tissue by rhBMP-2.

sity of the gelatin. This bulk density dependency could be elucidated that high density gelatin is relatively slow to degradate in vivo and possibly retain the residual rhBMP-2 longer in the device, resulting in the improvement of the performance, suggesting that gelatin is a good reservoir for control release of rhBMP-2.

In conclusion, implants of the new rhBMP-2 carrier PGS induce bone formation within their shape and it comprises one of the most promising osteogenetic biomaterials thus far demonstrated.

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