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Experimental Spinal Fusion With Recombinant Human Bone Morphogenetic Protein-2 Delivered by a Synthetic Polymer and β-Tricalcium Phosphate in a Rabbit Model

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Study Design. An experimental animal study to achieve posterolateral intertransverse process spine fusion with recombinant bone morphogenetic protein in combination with a new delivery system.

Objective. To evaluate the efficacy of a new synthetic biodegradable bone-inducing material containing recombinant human bone morphogenetic protein-2 (rhBMP-2) as a bone-graft substitute for posterolateral intertransverse process fusion in a rabbit model.

Summary of Background Data. rhBMP-2, a powerful bone-inducing cytokine, has been used as a bone graft substitute in combination with animal-derived collagen to achieve spinal fusion in animal models. However, the minimum dose of rhBMP-2 required to obtain solid posterolateral intertransverse process fusion was high on the basis of previous reports (>100 μg in rabbit models). To improve the efficacy, performance of rhBMP-2, and the safety of the delivery system for this protein, a more sophisticated system is required.

Methods. To fabricate one implant for one-side L4–L5 intertransverse process fusion, β-tricalcium phosphate (β-TCP) powder (300 μg), a polymer gel (PLA-DX-PEG block copolymer; 300 μg) and rhBMP-2 (7.5, 15, or 30 μg) were mixed and manually shaped to resemble a rod. Through a posterolateral approach, two implants were placed on both sides (1 per side) by surgery so as to bridge the transverse processes of adult New Zealand white rabbits (n = 27). In control animals, implants without rhBMP or autogenous cortico-cancellous bone chips from the iliac crest were placed in a similar location. The lumbar vertebrae were recovered 6 weeks after surgery. The posterolateral fusion was examined by manual palpation, radiography, biomechanical testing, and histology.

Results. Rabbits that received 15 or 30 μg of rhBMP-2 showed consistent fusion. However, solid fusion was seen in 2 of 5 rabbits with autografting and rabbits that received 7.5 μg of rhBMP-2. Fusion was not observed in the rabbits that did not receive rhBMP-2.

Conclusions. Consistent spinal fusion was obtained by implanting a biodegradable bone-inducing implant composed of β-TCP, PLA-DX-PEG, and rhBMP-2 within a period of 6 weeks. The rhBMP-2 doses required for the spinal fusion were significantly lower than those reported previously.

Key words: animal model, bone induction, posterolateral lumbar spine fusion, recombinant human bone morphogenetic protein-2. **Spine 2005;30:1717–1722**

Anterior or posterior fusion with autogenous bone grafting is a routine method for the treatment of spinal disorders associated with spinal instability resulting from degenerative changes, tumor resection, or trauma to the spine. To restore permanent stability of the spine, local new bone formation bridging the neighboring unstable vertebrae is essential. Autogenous iliac bone grafting is commonly used to promote bone formation. However, autogenous bone grafting is limited by some issues that remained unsolved. These are physical or cosmetic morbidities such as acute and chronic pain or dysesthesia, the potential risk for wound infection, extensive skin scarring, and deformity at the donor site.[1,2] In addition, the limited available mass of graft bone is also a disadvantage. To overcome these issues, new methods or materials that can substitute for the autogenous bone grafts have been desired. Allogeneic bone graft or banked bone is one of the alternatives that have been considered. However, banked bone has less osteogenic potential than autograft, and there is a potential risk for immunologic reaction from hosts and disease transfer to host with this material.[3,4] Biomaterials such as hydroxyapatite and bioactive ceramics also have been tested as bone-graft substitutes to avoid the potential risks arising from the use of allografts. Unfortunately, materials with osteoconductive potential but no osteoinductive capacity cannot substitute for autograft. Therefore, new materials

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with a potent osteoinductive capacity are required to avoid the disadvantages of autograft and to secure enhanced new bone formation for solid spinal fusion.

To manufacture an osteoinductive artificial bone graft substitute, cytokines retaining osteoinductive activity (bone morphogenetic proteins, BMPs) have been combined with biocompatible implant materials and used to obtain spinal fusion in experimental animals or in limited number of human cases.[5–15] To elicit the BMP-induced bone formation, a carrier material that delivers BMP slowly to the target cells is essential. As a carrier material, animal-derived collagen has been used routinely both in animal experiments and in clinical settings despite the potential risks for immunologic reaction in the host and transfer of diseases such as bovine spongiform encephalopathy (BSE).[16,17] To avoid those risks, we synthesized biodegradable polymers which work more effectively as the carrier for BMP-2 in *in vivo* conditions than bovine-derived collagen.[18,19] By use of this BMP delivery system, critical size defects in the long bones of rabbits and dogs were repaired successfully. New bone formation was achieved with these new porous solid biomaterials, which remained unresorbed in hosts.[20,21]

In this study, we attempted to achieve posterolateral intertransverse process spine fusion in the rabbit model by use of a biodegradable polymer and β-TCP composite as a delivery system for BMP. In this system, a successful outcome would be bone formation and the complete resorption of the carrier materials at the implanted sites.

Materials and Methods

rhBMP-2. rhBMP-2 was produced at Genetics Institute (Cambridge, MA) and donated to us through Astellas Pharma Inc. (Ibaraki, Japan).

PLA-DX-PEG Polymer. Poly-D,L-lactic acid with a random insertion of *p*-dioxanone/polyethylene glycol block copolymer (PLA-DX-PEG, MW; 12,400, LA/DX/EO molar ratio; 42:14:44), was provided by Taki Chemical (Kakogawa, Japan). The chemical formula of the PLA-DX-PEG is shown in Figure 1. We have reported that this polymer worked effectively as a carrier for rhBMP in previous studies. Details of the physicochemical characteristics and efficacy as a carrier material for rhBMP-2 have been reported elsewhere.[18,19] The minimal efficacious content of rhBMP-2 in the synthetic polymer required to elicit new bone formation in rabbits was approximately 0.02%.[20]

β-TCP Powder. β-TCP powder (less than 100 μm in diameter of particles) was manufactured and provided to us by Olympus Biomaterial (Tokyo, Japan).

Preparation of New Bone Graft Substitute Implants. To prepare one implant (Figure 2A) to bridge L5 and L6 transverse processes on one side, 300 mg of β-TCP powder, 300 mg of PLA-DX-PEG, and 3 dosages of rhBMP (7.5, 15, or 30 μg) were mixed and stirred with a metal rod at 50°C for several minutes. The resultant dough was then cooled and fabricated by hand to resemble a rod. The hardened rods were stored at −30°C until implantation. As control implants, 300 mg of β-TCP powder and 300 mg of PLA-DX-PEG without rhBMP-2 was prepared in the same manner.

$$H\text{-}(\text{-O-CH(CH}_3\text{)-CO-})_m\text{-}(\text{-O-CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CO-})_n\text{-O-}(\text{-CH}_2\text{-CH}_2\text{-O-})_o\text{-}(\text{-OC-CH}_2\text{-O-CH}_2\text{-CH}_2\text{-O-})_p\text{-}(\text{-OC-CH(CH}_3\text{)-O-})_q\text{-H}$$

PLA-DX Random polymer segment PEG Segment PLA-DX Random polymer segment

Figure 1. Structural formula of PLA-DX-PEG. The subscripts m, n, o, p, q represent variable number of units.

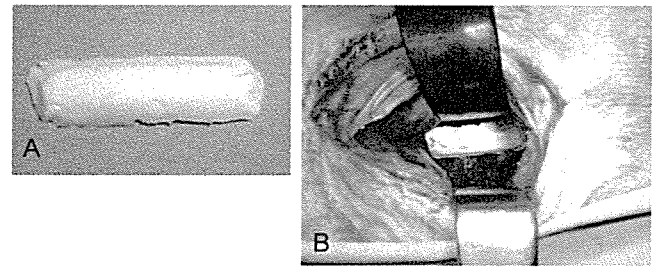

Figure 2. **A**, Prepared β-TCP dough implant. **B**, β-TCP dough was placed on the L5–L6 intertransverse region.

Surgery and Experimental Protocols. Twenty-seven New Zealand white rabbits (age, 1–2 years-old; weight, 3.5–4.5 kg) were divided randomly into five groups depending on the material to be implanted into the intertransverse process space. Before surgery, the animals were anesthetized with an intramuscular injection of ketamine (30 mg/kg) and xylazine (10 mg/kg). Cefazolin (100 mg) was administered subcutaneously as a prophylactic antibiotic. Each rabbit underwent surgery for a single level posterolateral intertransverse process fusion at L5–L6.[7] A dorsal midline skin incision was made, followed by two paramedian fascial incisions. The intermuscular plane between the multifidus and longissimus muscles was retracted to expose the transverse processes of L5 and L6 and the intertransverse membrane. An electric-driven burr (Stryker, Kalamazoo, MI) was used to decorticate posterior cortex of the respective transverse process, and one of the implant or transplant materials listed in Table 1 was implanted (Figure 2B). The wounds were then closed with 3–0 absorbable and 3–0 nylon sutures. Cefazolin (100-mg once daily) was administered to the respective animal subcutaneously for 3 days after surgery. The animals were killed by overdose of anesthetics at 6 weeks after surgery, and the L4-L7 lumbar spines were harvested and processed for further examination. This protocol was approved by the Institutional Committee for Animal Care and Experiments of Osaka City University Medical School.

Radiographic Evaluation. The L5–L6 spines from each group animals were examined by posteroanterior plain radio-

Table 1. Implant Assignement

Group	rhBMP-2 (μg)	β-TCP (mg)	PLA-DX-PEG (mg)	Concentration of rhBMP-2 (wt%)	n
BMP 30	30	300	300	0.005	5
BMP 15	15	300	300	0.0025	6 *
BMP 7.5	7.5	300	300	0.00125	5
BMP 0	0	300	300	0	6 *
Autogenous bone	Autogenous iliac bone graft (1–1.5 g)				5

* Each one is for histological evaluation.

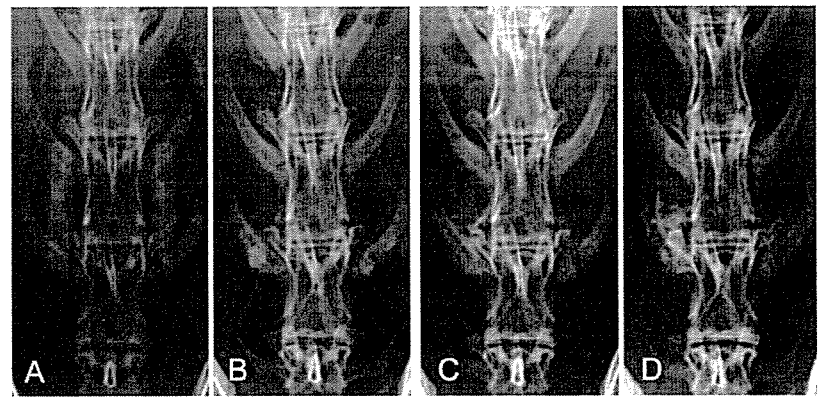

Figure 3. Representative posteroanterior radiographs of rabbit spines in the BMP 30 group, immediately after surgery (**A**), at 2 weeks (**B**), at 4 weeks (**C**), and at 6 weeks (**D**). In the radiograph, a remarkable fusion mass is visible between the L5–L6 transverse process at 6 weeks.

graphs and computed tomographic (CT) scans (GE Yokogawa Medical System, Tokyo, Japan) sequentially at 2, 4, and 6 weeks after surgery. CT images of 2-mm slice thickness were used to construct three-dimensional images. From the harvested lumbar spine samples, soft tissues were removed, and plain radiographs and CT images were taken again. A radiologic evaluation for intertransverse process fusion was made by three observers in a blinded fashion. Fusion was graded as solid when two of the three observers agreed that the presence and location of new bone formation were consistent with a successful fusion outcome.

Manual Palpation. The harvested lumbar spines were manually palpated by flexion and extension at the fusion level and comparison with the adjacent level. Each motion segment was graded as solid fusion or not solid.

Biomechanical Testing. Biomechanical testing to evaluate the solidity of the L5–L6 fusion site was performed by a three-point flexion-bending test using a materials testing machine (Instron 5882, Instron, Boston, MA). Three-point bending tests were performed with a 30-mm intersupport distance and a 1 mm/minute head speed. The bending moment at 1.5-mm middle-span deflection was determined from the moment-deflection curves.

Histologic Examination. The harvested specimens were fixed in 10% formalin in neutral buffer solution and decalcified in 10% formic acid solution, dehydrated in a gradient ethanol series, and embedded in paraffin. Sections of 4-μm thickness at the intertransverse process region were cut in a sagittal plane, stained with hematoxylin and eosin, and observed under a light microscope to examine for bony fusion between the newly formed bone and the original transverse processes.

Statistical Analysis. Comparisons of biomechanical testing of spines in each group were made using one-way analysis of variance. The post hoc Sceffe test was performed to determine significant differences between groups. Significance for all tests was defined as $P < 0.05$.

Results

Radiographic Evaluation

An opaque shadow of β-TCP was noted at the operated site on radiographs immediately after surgery. Radiographs at 6 weeks showed homogeneous calcified shadows between the transverse processes in all animals of the BMP15, BMP30, and in part of the BMP7.5 groups-.(Figures 3–5) Representative three-dimensional CT images of each group are shown in Figure 6. Fusion assessments in three-dimensional CT were difficult because the images tend to overestimate the fusion mass. Results of the evaluation from plain radiographs and three-dimensional CT are shown in Table 2.

Manual Palpation

In all samples of the BMP 15 and BMP 30 groups, bony hard masses at the intertransverse process were palpable, and the passive motion between the vertebrae was significantly restricted when compared with that in control samples. Two of the five samples from the BMP 7.5 and autogenous bone groups, respectively, were evaluated as

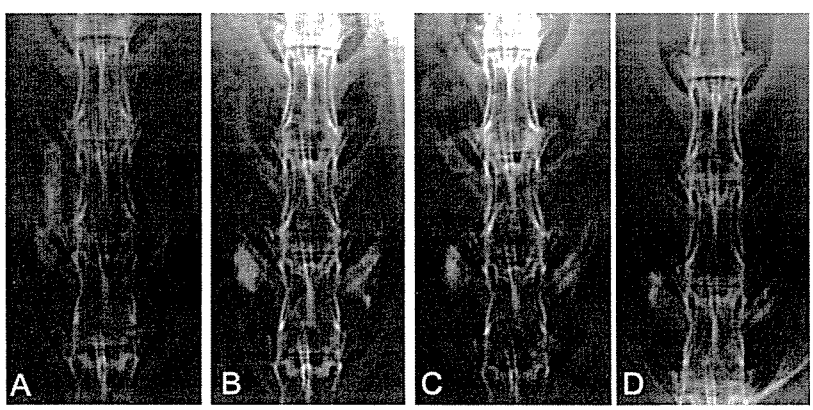

Figure 4. Representative posteroanterior radiographs of rabbit spines in the BMP 0 group, immediately after surgery (**A**), at 2 weeks (**B**), at 4 weeks (**C**), and at 6 weeks (**D**). In the radiograph, no fusion mass between the L5–L6 transverse process is visible.

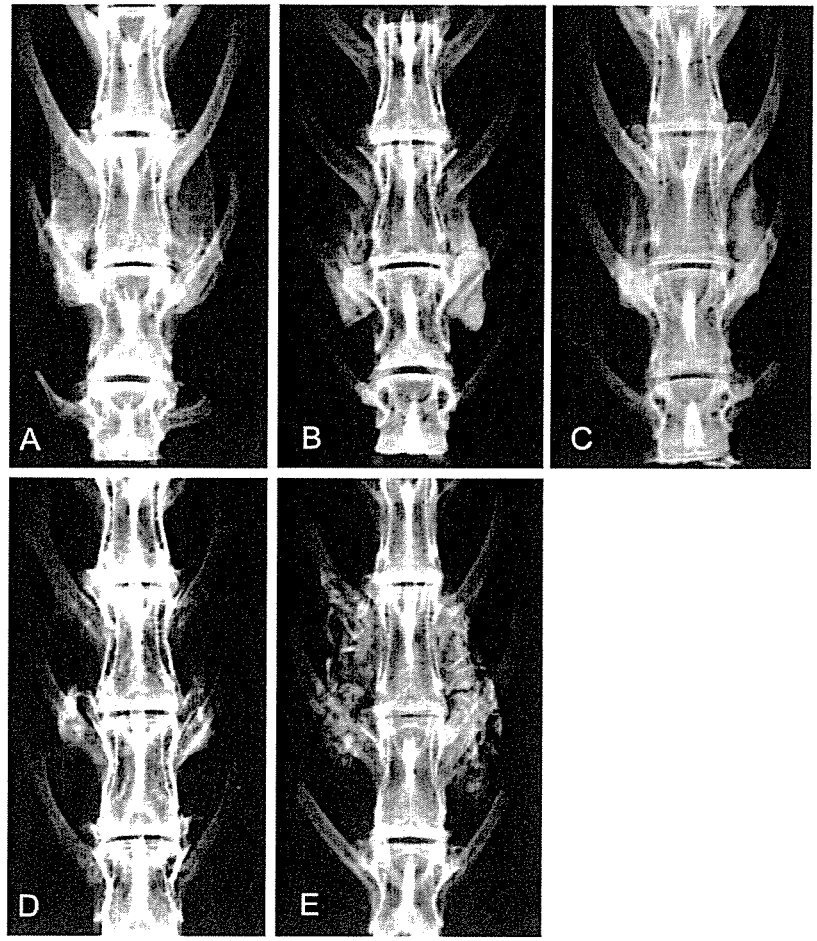

Figure 5. Harvested lumbar spine 6 weeks after surgery. BMP30 (**A**), BMP15 (**B**), BMP7.5 (**C**), BMP0 (**D**), autogenous bone (**E**).

solid fusion outcomes. In contrast, none of the samples from the BMP 0 groups achieved solid fusion (Table 3).

Biomechanical Testing

The results from biomechanical testing in each of the experimental groups are shown in Figure 7. The bending moment at 1.5-mm middle-span deflection of the BMP 15 and BMP 30 groups were significantly larger than the BMP 0 group. The mean values in the BMP30 and BMP15 groups were higher than those from the autogenous bone group and the control BMP 0 group.

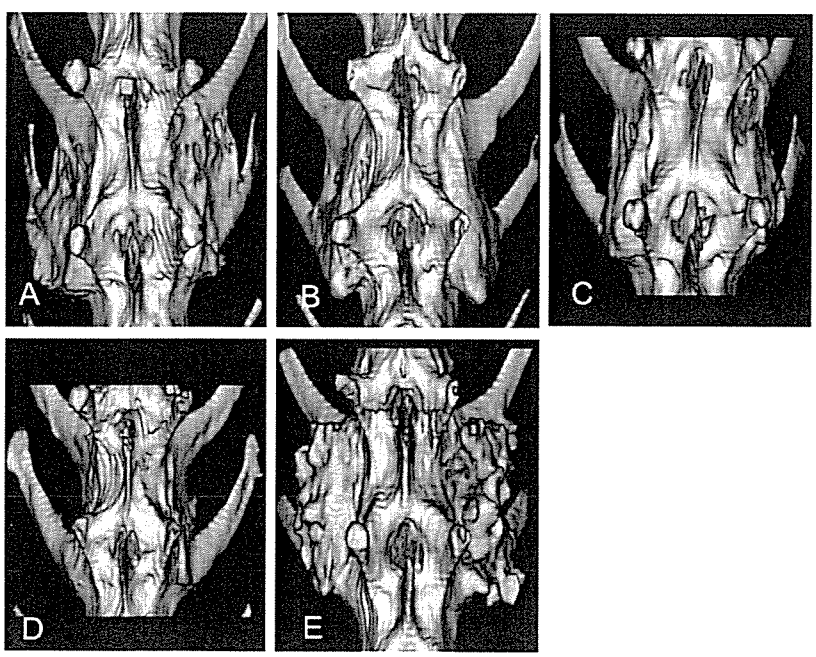

Figure 6. Three-dimensional-CT at 6 weeks after surgery. BMP30 (**A**), BMP15 (**B**), BMP7.5 (**C**), BMP0 (**D**), autogenous bone (**E**).

Table 2. Fusion Rate in Radiological Assessments

Group	Plain X-p	Three-Dimensional CT
BMP 30	5/5	5/5
BMP 15	5/5	5/5
BMP 7.5	4/5	4/5
BMP 0	0/5	0/5
Autogenous bone	5/5	5/5

Histologic Examination

Lower magnification views of sagittal sections (hematoxylin and eosin staining) of fusion mass in animals that received rhBMP-2 showed bone mass with peripheral cortical bone bridging the transverse processes. Higher magnification of the bridging bone mass revealed woven bone and hematopoietic marrow. Tiny remnants of the β-TCP powder were also recognized. However, the intertransverse region sampled in a spine that did not receive rhBMP-2 revealed fibrous tissue and remnants of β-TCP with no evidence of new bone formation (Figure 8).

Discussion

The present study was designed to test a synthetic and absorbable bone-graft substitute with osteoinductive ability equivalent or superior to the autogenous bone graft. The test was conducted in a critical bone defect model wherein the successful outcome was a solid posterolateral intertransverse process fusion. The results in this study were satisfactory, and in all animals with β-TCP (300 mg)/PLA-DX-PEG (300 mg) composite implants with 15 or 30 μg of rhBMP-2, solid spinal fusion was obtained in 6 weeks. In the autogenous bone graft group, new bone formation was consistently recognized on radiographs, but in some specimens retrieved from the rabbits, failure of fusion was noted during biomechanical tests. It is interesting to note that in some clinical cases, the pseudarthrosis rate of posterolateral spine fusion has ranged from 5 to 35%.[22,23] These results are encouraging and point to the need for further clinical testing of this synthetic composite implant. The avoidance of additional surgery to harvest graft bone and thereby eliminate donor site morbidities is a potential key benefit of this approach.

In considering the practical application of the rhBMP retaining implants, one of the remaining issues is the extremely high dose of rhBMP required to elicit new bone, especially in humans. Typically, several milligrams of rhBMP are necessary for one level spinal fusion, and this fact results in a high cost of the BMP-retaining implant.[6] To reduce the cost, a more effective method to deliver efficacious but lower doses of rhBMP-2 would be desired. In previous experimental studies of the rabbit posterolateral intertransverse process spine fusion model, >100 μg of rhBMP-2 were required to achieve the solid one-level spine fusion in 5 or 6 weeks. This was accomplished by the use of either animal-derived collagen sheets, hydroxyapatite with collagen, an open cell polylactic acid polymer, or sintered bovine bone as the delivery system for rhBMP-2.[5,10,12–15] Interestingly, in this study, the minimal effective dose of rhBMP-2 was significantly lower (15–30 μg) than the amounts used in previous studies. Those prior experiments were not reproduced in this study; therefore, a direct comparison of the minimal effective dose of rhBMP is difficult. However, our results suggest an advantage of this new delivery system to reduce the effective dose of rhBMP for spinal fusion. The use of β-TCP powder to construct the bone-inducing implants provides three advantages; 1) the addition of the β-TCP powder to the "sticky" PLA-DX-PEG resulted in a dough-like material with easy handling and molding characteristics, 2) a volume expansion of the implant by addition of β-TCP powder, and 3) resorption of the β-TCP powder by osteoclasts recruited during the BMP-induced bone formation phase and disappears after establishment of spinal fusion. In our previous study in mice, it was noted that the β-TCP granules coated with the rhBMP-2-retaining polymer (PLA-DX-PEG) elicited ectopic new bone in situ at 3 weeks. β-TCP encased within the ectopic ossicles was resorbed by a large population of osteoclasts attached on the surface.[24] As expected in the current study, the β-TCP powder was almost completely resorbed and replaced by new bone with marrow on histologic findings at 6 weeks after surgery.

In summary, a new bone-inducing and biodegradable implant was produced by combining three synthetic materials (PLA-DX-PEG, rhBMP-2, and β-TCP). Posterolateral intertransverse process fusion was successfully achieved in a rabbit model with this implant in 6 weeks. The fusion rate appeared to exceed that obtained by autogenous bone grafting. These data provide support for the use of this new biomaterial as a substitute for autogenous bone grafting. The avoidance of the need for and

Table 3. Fusion Rate in Manual Palpation

Group	Solid Fusion
BMP 30	5/5
BMP 15	5/5
BMP 7.5	2/5
BMP 0	0/5
Autogenous bone	2/5

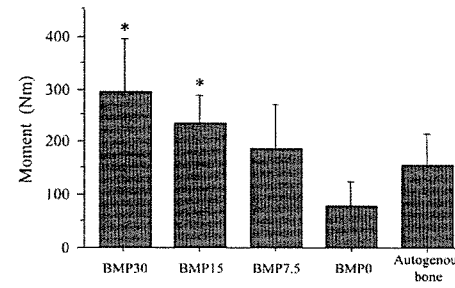

Figure 7. Results of three-point flexion bending tests for each specimen. These results indicate the fusion sites of BMP 30 and BMP 15 groups are stiffer than BMP 0 group. *, statistically significant difference from BMP 0 group.

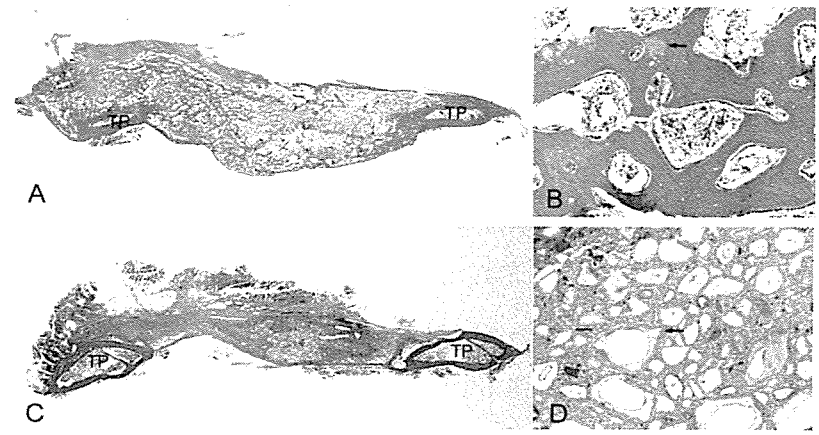

Figure 8. Hematoxylin and eosin stained sagittal section of L5–L6 intertransverse region in BMP 15 (**A,B**) and BMP 0 (**C,D**) group. New bone formation between the L5–L6 transverse process (TP), the cortical rim around fusion mass, and tiny residual β-TCP (arrow) was seen in BMP 15 group. The specimen from the group that did not receive rh-BMP-2 showed no new bone formation between the L5–L6 transverse process (**A and C**, ×0.5, **B and D**, ×10).

risks associated with surgery for graft material procurement is an additional benefit. Additional preclinical study involving nonhuman primates will be required to evaluate the utility and safety of this implant for spine fusion.

Key Points

- The efficacy of the β-TCP/PLA-DX-PEG composite that contained low doses of rhBMP-2 was evaluated in a New Zealand White rabbit posterolateral lumbar intertransverse process fusion model.
- Rabbits that received 15 or 30 μg of rhBMP-2 on each side, which is a significantly lower efficacious dose than previously reported, achieved solid fusion within 6 weeks in all experimental animals.
- This new synthetic biodegradable bone-inducing material could reduce the dose of rhBMP-2 required to achieve solid fusion with no residual trace of the implant.
- The current study demonstrated the potential of the new material as a substitute for autogenous bone graft material in spine fusion. This material obviates the need for bone graft procurement and thereby reduces the risk of morbidity often associated with this surgery.

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Repair of an intercalated long bone defect with a synthetic biodegradable bone-inducing implant

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Abstract

Recombinant human bone morphogenetic protein (rhBMP)-2 in a block copolymer composed of poly-D,L-lactic acid with randomly inserted *p*-dioxanone and polyethylene glycol (PLA-DX-PEG) as a carrier and porous β-tricalcium phosphate (β-TCP) blocks were used to generate a new fully absorbable osteogenic biomaterial. The bone regenerability of the rhBMP-2/PLA-DX-PEG/β-TCP composite was studied in a critical-sized rabbit bone defect model. In an initial study, a composite of PLA-DX-PEG (250 mg) and β-TCP (300 mg) loaded with or without rhBMP2 (50 μg) was implanted into a 1.5 cm intercalated bone defect created in a rabbit femur. Defects were assessed by biweekly radiography until 8 weeks postoperatively. The bony union of the defect was recognized only in the BMP-loaded group. To obtain further data on biomechanical and remodeling properties, another BMP-loaded composites group was made and observed up to 24 weeks. All defects were completely repaired without residual traces of implants. Anatomical and mechanical properties of the repaired bone examined by histology, 3-dimensional CT (3D-CT) and mechanical testing were essentially equivalent to the nonoperated-on femur at 24 weeks. These experimental results indicate that fully absorbable rhBMP-2/PLA-DX-PEG/β-TCP is a promising composite having osteogenicity efficient enough for repairing large bone defects.

Keywords: BMP (bone morphogenetic protein); Bone tissue engineering; Biodegradation; Osteoconduction; Drug delivery; Calcium phosphate

1. Introduction

The long history of orthopedic practice has confirmed the efficacy of autogenous bone grafting, but significant problems such as donor site morbidity and the limited supply associated with autogenous bone harvesting remain. To overcome these problems, a variety of osteoconductive biomaterials, e.g., ceramics and titanium alloys, have been considered as bone graft substitutes [1,2]. Experience to date with osteoconductive biomaterials suggests that these can be made in greater quantities, would improve biomechanical strength, and present no concerns with immunogenicity [3]. These osteoconductive biomaterials are already efficacious in repairing unloaded bone cavities or small bone defects where an efficient osteogenic cell supply can be anticipated. Repairing large loaded bone defects using osteoconductive biomaterials alone remains a challenge, however, because osteogenic cells are not likely to be recruited without osteoinductivity in the center of large defects, and the lack of absorbability presents an obstacle to remodeling necessary to withstand repetitive mechanical loading. These considerations led us to conclude that biomaterials used to repair large bone

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defects should be completely biodegradable and have good osteoconduction and osteoinduction.

Among biodegradable and osteoconductive biomaterials such as synthetic porous polymers (poly[L-lactide-coglycolide] copolymer (PLGA)) and tricalcium phosphates, porous beta-tricalcium phosphate (β-TCP) has proved most popular in current orthopedic surgery. Porous β-TCP has interconnected pores, which aid in infiltrating osteogenic cells, and is strong enough to maintain implant shape during bone formation [4]. β-TCP, as well as natural bone matrix, is mainly absorbed by cells positive for tartrate-resistant acid phosphatase (TRAP) and is replaced by newly formed bone. Reports have also confirmed its osteoconductivity and degradability at orthotopic sites [5].

Osteoinductivity is currently added to biomaterials three main ways: (1) applying cultured osteogenic cells from autologous bone marrow (cell-based), (2) applying osteoinductive cytokines (cytokine-based), and (3) applying osteoinductive genes [6–8]. We used the cytokine-based approach because of its promising clinical application since several human osteoinductive cytokines have been produced by recombinant techniques [9].

Bone morphogenetic proteins (BMPs) are biologically active osteoinductive cytokines with significant clinical potential, but the lack of a delivery system enabling full osteoinduction has precluded their wider implementation in clinical therapeutics [10]. Ideal delivery should be synthetic to avoid the disease transmission possible with allogenic materials. Current carrier materials for BMPs, such as collagen sponges and hydroxyapatite, show successful bone formation in vivo, but require a high BMP dose because of their inability to retain BMPs [11,12]. Another requirement for ideal delivery is thus controlled BMP release enabling the amount of BMPs required for bone repair to be reduced by ensuring effective BMP retention in reactive cells. For this, we developed a synthetic block copolymer composed of poly-D,L-lactic acid with randomly inserted *p*-dioxanone and polyethylene glycol (PLA-DX-PEG), reported to deliver BMPs effectively [13–15].

To this end, we used porous β-TCP and recombinant human BMP-2 (rhBMP-2) with PLA-DX-PEG to generate a new, fully absorbable osteogenic biomaterial for repairing loaded large bone defects. We evaluated the efficacy of rhBMP-2/PLA-DX-PEG/β-TCP composite in bone induction and degradability using a critical-sized intercalated rabbit bone defect model.

2. Materials and methods

2.1. Materials

Porous β-TCP cylinders of OSferion® 6 mm in diameter, 5 mm high, and weighing approximately 100 mg were manufactured and donated (Olympus Biomaterials Co., Ltd., Tokyo, Japan) for the purpose of these studies. Pores were 100–400 μm in size, porosity was 75%, and the sintering was at 1050° [4]. rhBMP-2 produced at the Genetics Institute (Cambridge, MA) and donated through Yamanouchi Pharmaceutical Co., Ltd. (Ibaraki, Japan), was dissolved in a buffer of 5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80 at a concentration of 1 μg/μl. As a rhBMP-2 delivery system, a block copolymer of poly-D,L-lactic acid with randomly inserted *p*-dioxanone and polyethylene glycol (PLA-DX-PEG; MW 9,600; PLA/DX/PEG molar ratio 43/14/43) was synthesized and provided by Taki Chemical Co., Ltd. (Kakogawa, Japan). The physicochemical properties and efficacy of the rhBMP-2 carrier material are detailed elsewhere [13–15]. We determined the minimal optimal rhBMP-2 content in the synthetic polymer required to elicit new bone formation to be approximately 0.02% (wt/wt) in rabbits from previous experiments [16–18].

2.2. Preparation of implants

To combine polymer and rhBMP-2, 250 mg of polymer was dissolved in 3 ml of distilled acetone and 50 μg of rhBMP in 0.01N HCl was mixed in a glass vial. Three porous β-TCP cylinders were then submerged in the mixed solution and placed in a vacuum for a few seconds to replace air in β-TCP cylinder pores with solvent. Acetone was then removed with a centrifuge evaporator. The glass vials were shaken several times during evaporation to thoroughly impregnate cylinders with the rhBMP-2 delivery material. Treated porous β-TCP cylinders were coated with the rhBMP-2/ PLA-DX-PEG composite (50 μg of rhBMP2, 250 mg of PLA-DX-PEG, and 300 mg of β-TCP (3 cylinders) for each defect). Implants were kept in a freezer at −30 °C until use. Control implants were β-TCP cylinders coated with PLA-DX-PEG without rhBMP-2 prepared the same way as above.

2.3. Scanning electron microscopy (SEM)

The surface of porous β-TCP blocks was observed by scanning electron microscopy (SEM; Hitachi 4700SI) to ensure that the pore surface was coated by rhBMP-2/ PLA-DX-PEG homogenously (Fig. 1).

2.4. Animals and operative procedures

Of 24 Japanese white rabbits 3 months old purchased from Japan SLC Co., Ltd. (Shizuoka, Japan), 15 were used in Study 1 and 9 in Study 2. All experiments were conducted strictly observing institutional guidelines for the care and use of laboratory animals.

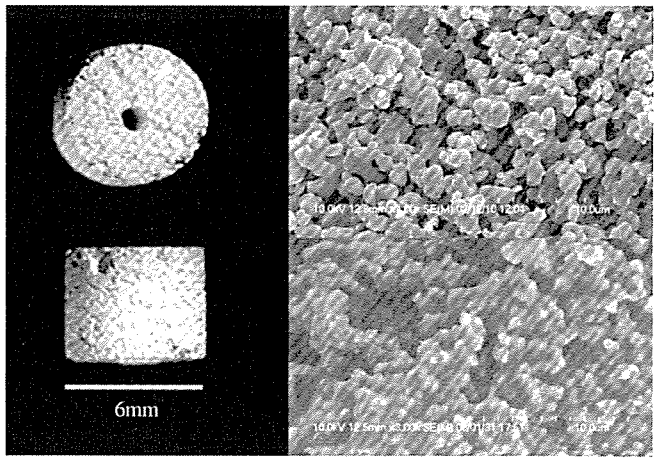

Fig. 1. Macroscopic aspect of β-TCP cylinders: images at left are axial and side views of the β-TCP cylinder, and at right are SEM micrographs of the β-TCP cylinder coated with PLA-DX-PEG ×3000 (above) and the noncoated control (×3000, below). The β-TCP cylinder consists of 1 μm sintered β-TCP granules with 100–400 μm interconnected pores. The surface of β-TCP granules in the experimental group is uniformly covered by a thin polymer layer.

Rabbits were anesthetized using an intramuscular injection of ketamine (60 mg/kg body weight) before surgery. Under sterile conditions, the mid-shaft of the femur was exposed through a lateral longitudinal skin incision and thigh muscles divided. A 1.5 cm long section of the diaphysis was removed together with the periosteum using a cutting saw and the bone defect either filled with prepared implants or left as is [19]. Femurs were then fixed with external fixators for up to 8 weeks postoperatively.

2.5. Experimental design

2.5.1. Effects of rhBMP-2/PLA-DX-PEG/β-TCPon bone defect repair (Study 1)

Fifteen rabbits were divided into 3 groups of 5 each based on implants received, i.e., (1) 50 μg of rhBMP2, 250 mg of PLA-DX-PEG, and 300 mg of β-TCP; (2) no rhBMP2 but the same amounts PLA-DX-PEG and β-TCP; and (3) no implant (defect controls). Femurs were assessed every 2 weeks using X-ray radiography, and bone formation was evaluated as shown in X-ray photographs below. All animals were euthanized by anesthesia overdose at 8 weeks and femurs in the first group collected and stored at −30 °C until mechanical testing in Study 2. β-TCP alone was not assessed in this study because PLA-DX-PEG alone only negligibly affected β-TCP osteoconductivity and degradability in previous studies [20].

2.5.2. Mechanical and remodeling properties of the repaired bone by rhBMP-2/PLA-DX-PEG/β-TCP with long-term observation (Study 2)

Five rabbits underwent the same implants as group (1) in Study 1, i.e., 50 μg of rhBMP2, 250 mg of PLA-DX-PEG, and 300 mg of β-TCP, and external fixators were removed at 8 weeks postoperatively and observed for 24 weeks until animals were euthanized as described above. Femurs were collected to examine bone mineral density, then mechanically tested together with the 5 specimens in Study 1. Four other rabbits undergoing implants of 50 μg of rhBMP2, 250 mg of PLA-DX-PEG, and 300 mg of β-TCP were euthanized (1 each) at 2, 4, 8, and 24 weeks postoperatively for histological examination.

2.6. X-ray analysis

New bone formation was evaluated by measuring the ratio of recognizable occupying callus to the defect in length because it is difficult to distinguish newly formed bone from β-TCP due to its radiopacity in the central portion of the defect [21].

2.7. Dual-energy X-ray absorptiometry analysis

Femurs harvested 24 weeks after surgery in Study 2 were examined via dual-energy X-ray absorptiometry (DXA) (DCS-600EX-III; Aloka Co., Ltd., Tokyo, Japan) using an analysis program designed for small animals. Bone mineral content (BMC) and area of new bone were measured and bone mineral density (BMD) calculated as BMC/bone area. Nonoperated-on femurs harvested from animals euthanized at 24 weeks were used as controls.

2.8. Mechanical testing

Five regenerated femurs each, harvested at 8 weeks in Study 1 and at 24 weeks, were used in 3-point bending mechanical testing with an apparatus designed for this purpose (Maruto Testing Machine Co., Tokyo, Japan). Nonoperated-on femurs harvested from animals euthanized at 24 weeks were used as controls. Other femurs implanted without rhBMP-2 in Study 1 showed no union, and hence were not mechanically tested. Maximum bending strength was measured.

2.9. Histological examination

For histological examination, samples were decalcified in 10% formic acid, dehydrated in a gradient ethanol series, mounted in paraffin, sectioned 4 μm thick, and stained with hematoxylin-eosin. To observe bone resorption, specimens were stained with tartrate-resistant acid phosphatase (TRAP). Briefly, deparaffinized sections were placed in a TRAP-staining solution consisting of acetate buffer (pH 5.0) 50 mM sodium tartrate, 25 mg/ml Naphthol-AS MX phosphate (Sigma Chemical Co., MO, USA), and 0.5 mg/ml

fast red violet salt (Sigma Chemical Co.) and incubated at 37 °C for 120 min. After the solution was removed by washing, specimens were counterstained with hematoxylin and observed under light microscopy.

2.10. Computed tomography (CT) scanning images

Computed tomography (CT) data on harvested distal femurs at 24 weeks was collected with a helical CT (GE Yokogawa, Tokyo, Japan) and images were reconstructed using 3-dimensional image reconstruction software (Aze, Tokyo, Japan).

2.11. Statistical analysis

Student's t-test was used to determine statistical significance, with $P<0.05$ considered significant.

3. Results

3.1. Defect repair effect of rhBMP-2/PLA-DX-PEG/β-TCP (Study 1)

Representative radiology of bone defects at 2, 4, and 8 weeks after surgery (Fig. 2) demonstrated opaque calcified shadows bridging both ends of defects as early as 2 weeks in the group implanted with β-TCP combined with rhBMP-2 and polymer. Calcification became more evident at 4 weeks, and newly formed bone connecting both ends of defects appeared to have been remodeled into cortical bone with a bone marrow cavity. The BMP-loaded group showed a time-dependent increase in callus of nearly 100% at 6 weeks, but the group with β-TCP and polymer without rhBMP-2 showed only small amounts of newly formed bone formation—less than 20%. No bone formation was recognized in the control group (Fig. 3).

3.2. Mechanical and remodeling properties of the repaired bone by rhBMP-2/PLA-DX-PEG/β-TCP with long-term observation (Study 2)

Femurs in this group were stable and enabled free movement in caged animals until their sacrifice at 24 weeks. Radiology of femurs harvested 24 weeks after surgery showed complete regeneration of the intercalated defect. The 3D CT image and frontal tomographic image of regenerated femurs showed that normal femur anatomy had been restored with cortical bone with no residual evidence of implanted β-TCP cylinder blocks (Fig. 4).

3.2.1. Dual-energy X-ray absorptiometry analysis

Bone mineral density of femurs regenerated by biodegradable bone-inducing implants at 24 weeks was 350 mg/cm^2, essentially equal to control (normal) levels. No significance was seen between experimental and control groups.

3.2.2. Biomechanical properties of repaired bone

The 3-point bending test of femurs regenerated by biodegradable bone-inducing implants at 8 weeks showed significantly lower stiffness (160 N/m) than

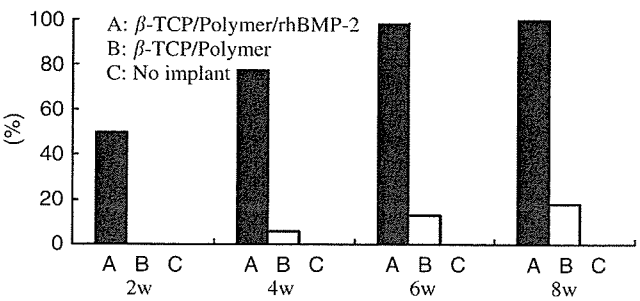

Fig. 3. BMP-loaded group promoting a time-dependent increase in callus (nearly 100% at 6 weeks). The group of β-TCP and polymer without rhBMP-2 promoted only negligible new bone formation—less than 20%. No bone formed in the control group.

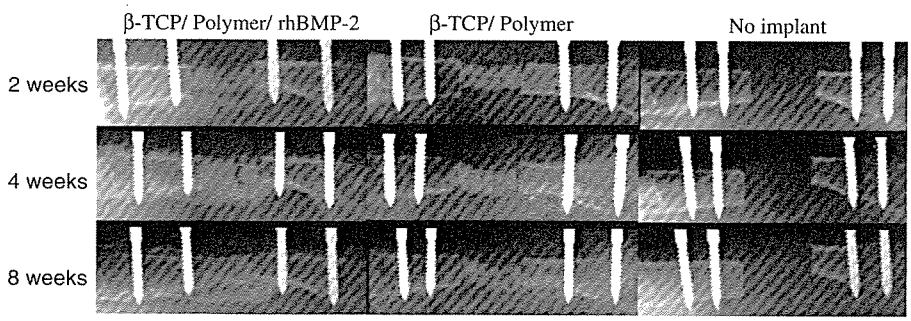

Fig. 2. Representative femur radiographs. From left, implanted with β-TCP with PLA-DX-PEG and rhBMP-2, β-TCP with PLA-DX-PEG without rhBMP-2, and critical size bone defect without implantation (sham surgery). Sequential radiographs show bone repair at 2, 4, and 8 weeks after implantation in the experimental group.

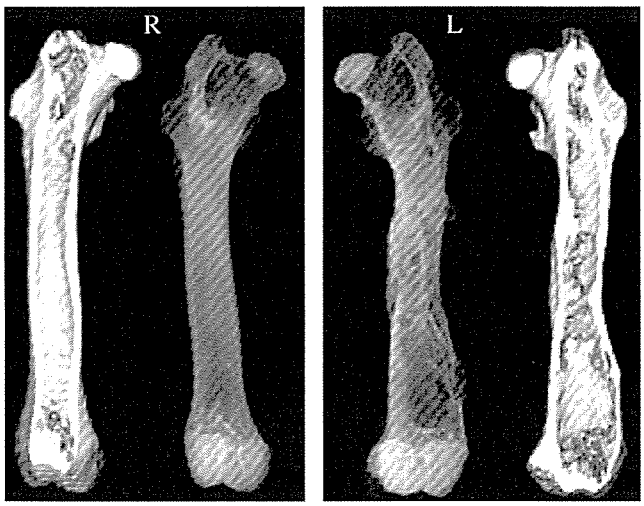

Fig. 4. Soft X-ray and 3D CT images of femurs 24 weeks after surgery. The repaired defect with biodegradable bone-inducing implants is shown at right (L). Images at left are of the counterpart femur in the control rabbit (R). The external fixator was removed 8 weeks after surgery. Note that β-TCP was absorbed and cortical walls remodeled anatomically with the marrow cavity.

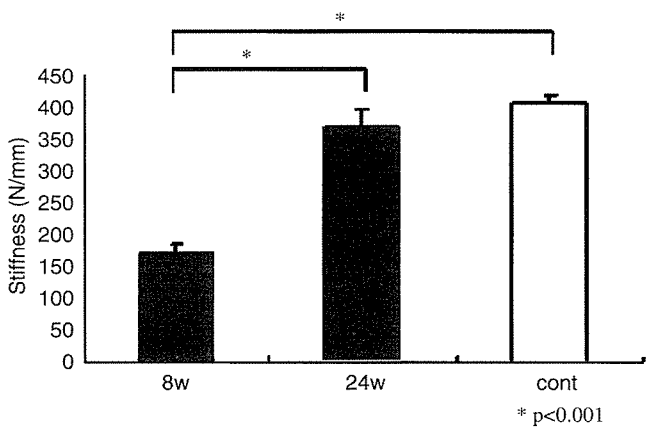

Fig. 5. Results of 3-point bending tests at 8 and 24 weeks after surgery (bar chart). (*: significant difference, $p<0.001$).

controls (400 N/m, nonsurgical femurs 24 weeks after surgery). Stiffness increased at 24 weeks (370 N/m) and was essentially equal to control (normal) levels (Fig. 5).

3.2.3. *Histological findings*

In histological sections from defect sites 2 weeks postoperatively, fibrous tissue and a thin layer of bone running parallel to the long axis and encasing implants were seen in the experimental group. A femur from the experimental group at 4 weeks with increased bone mass connected to β-TCP implants and fibrous tissue was observed. Regenerated cortical bone had united with ends of the original cortex of the femur. In sections from the experimental group 8 weeks postoperatively, bone occupying defects had remodeled to where cortical bone and hematopoietic marrow-like tissue were clearly visible (Fig. 6). On the β-TCP surface at 4 weeks, large numbers of TRAP-positive multinucleated cells (osteoclasts) appeared (Fig. 7). In sections of regenerated defects at 24 weeks, cortical bone was remodeled to lamellar bone connected to original ends of the femur. Marrow tissue was also completely restored and no remnants of β-TCP implants were visible (Fig. 8).

4. Discussion

Our experimental results indicated successful regeneration of a critical intercalated bone defect in femur implantation using porous β-TCP with rhBMP-2 and a synthetic PLA-DX-PEG block copolymer as its delivery system. This approach clearly demonstrated that combining these synthetic materials and recombinant protein repaired large defects. The osteogenic potential of composite implants has yet to be compared critically, however.

The rhBMP-2 dose and rhBMP-2 concentration in polymer we used was determined based on previous study of critical bone defect repair in rabbits in which 1.5 cm of an intercalated bone defect in the humerus was

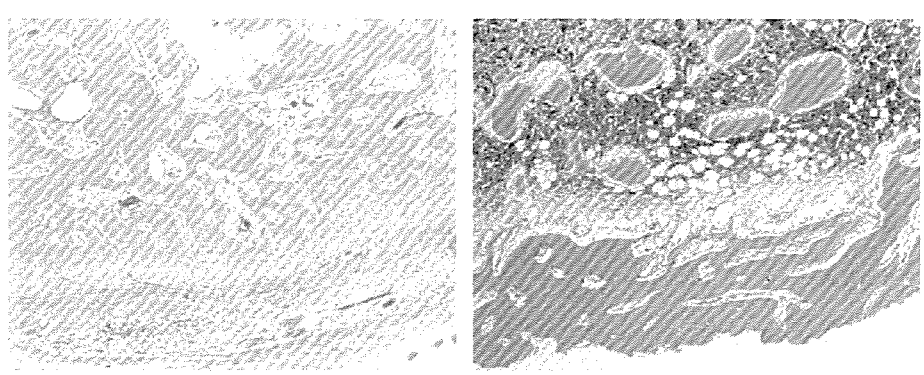

Fig. 6. Typical histological sections at 4 weeks (left, HE staining × 40) and 8 weeks (right, HE staining × 40). Abundant bone formed around β-TCP but not the outside of the femur in sections of specimens at 4 weeks. Cortical and bone marrow-like tissue clearly identified at 8 weeks.

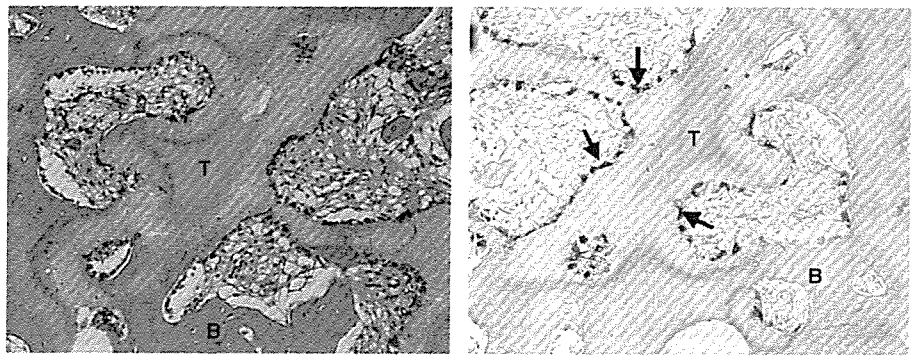

Fig. 7. Histological section of decalcified specimens harvested at 4 weeks and stained with HE (left, ×40) and tartrate-resistant acid phosphatase (TRAP) (right, ×40). β-TCP blocks coated with PLA-DX-PEG and rhBMP-2 are surrounded by abundant TRAP-positive multinucleated osteoclasts (arrows, stained red). (B = bone, T = β-TCP).

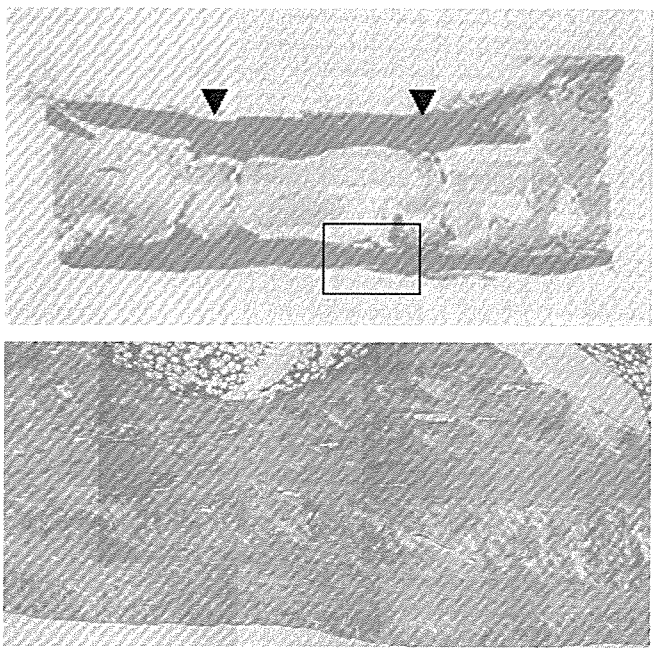

Fig. 8. Images of sagittal histological section at 24 weeks. Cortical walls were repaired completely. Arrowheads indicate fixator pin insertion locations. Polarized magnified image of junction between original and repaired bone (rectangle) (below). Randomly arrayed collagen in the repaired cortical wall contrasts with the more ordered layers of collagen in the original cortical wall.

successfully repaired by filling the defect with titanium mesh cylinders impregnated with a delivery system containing rhBMP-2 [17]. Titanium cylinders were not resorbed in repaired bone. Sustained permanent release of metal ions from the implant could increase the potential risk of allergic reactions or carcinogenesis, especially in infants. For these reasons, we replaced titanium in the present study with biodegradable material. As expected, β-TCP was completely resorbed and replaced by host bone within 24 weeks with no apparent adverse events from resorbed β-TCP.

The successful regeneration of the critical bone defect may be due in part to the porosity of β-TCP cylinders having 100–400 μm pores, which may enable rhBMP to accumulate locally within pores to where osteogenesis is initiated [22–25]. An appropriate local rhBMP concentration in pores would in turn enable successful invasion and ingrowth of mesenchymal cells in the implant and subsequent differentiation into osteoblasts. Additionally, the porosity of β-TCP appeared to be crucial to its rapid degradation in host animals, facilitating contact with host cells and resulting clearly in large numbers of osteoclasts contacting β-TCP [26]. The ability of these cells to permeate and resorb the β-TCP mass likely contributed to the relatively rapid replacement of implants by bone and marrow in our study. Although the location of BMP receptors on the surface of osteoclasts was reported previously [27], the effect of rhBMP-2 on osteoclastic differentiation remains to be clarified, requiring further study to determine the potential action of BMP on osteoclasts to explain the increased recruitment of osteoclasts in new bone induced by rhBMP2. Biodegradable osteoconductive β-TCP combined with a BMP delivery system is replaced by fully integrated biomechanically competent bone, eliminating one of the major limitations of other osteoconductive biomaterials.

The newly formed bone repairing the defect was remodeled to restore the normal anatomy of the original bone with concurrent resorption of β-TCP in 8 weeks. This means that BMP-induced new bone could remodel to adapt to the local biomechanical environment. Osteoconductive material must disappear to generate the physiological biomechanical environment and restore the original anatomy. Our results suggest that absorbable β-TCP is suitable as an ideal bone graft substitute.

The physicochemical properties and degradation profiles of polymer used in this study have been reported elsewhere [13–15]. A detailed safety check for clinical use of the polymer is currently in process. No systemic or local adverse effects have been noted to date.

The optimal content of rhBMP-2 in the carrier required to elicit new bone formation depends on the animal host [16–18], and a higher dose of rhBMP-2 is required in highly evolved animal species. Determining the optimal clinical dose thus requires additional experiments in primates.

5. Conclusion

A new absorbable bone graft substitute with osteogenic capacity was made by combining 3 artificial materials—porous β-TCP, rhBMP-2, and a PLA-DX-PEG delivery system. The capacity of this composite implant to regenerate bone is satisfactory. The composite implant was completely absorbed and replaced by newly formed bone, then remodeled into the femur to restore the natural anatomy. Although further safety checks and clinical trials are required, the practical use of this implant to promote bone regeneration is without doubt a realistic possibility.

Acknowledgements

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V．骨粗鬆症の治療

骨粗鬆症性椎体骨折に対する椎体形成術の現状

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骨粗鬆症性椎体骨折に対する椎体形成術は，損傷椎体の強度を保持あるいは増強させ，早期に疼痛を軽減させる手技として注目されている。通常はpolymethylmethacrylate（PMMA）骨セメントやcalcium phospate cement（CPC）等の骨セメントが使用される。この際，椎体外へのセメントのリークあるいはそれに続発する神経合併症や肺塞栓症が問題となる。

これらの問題点を解消するために，あらかじめ椎体内に十分な空間を作成しておくことが必要で，我々はバルーンによる椎体内空隙形成および内視鏡下掻爬を応用した，椎体形成術を偽関節例に対して施行している。本方法により疼痛は著明に軽快し，それに伴い日常生活動作（ADL）の改善が認められた。また肺塞栓症を代表とする全身合併症は認められなかった。今後の展望として，受傷後の予後不良因子を検討し，不良因子を有する症例に対してはより早期に本法を施行する必要がある。

Vertebroplasty for osteoporotic vertebral fracture

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Vertebroplasty for osteoporotic vertebral fracture has been published to enhance fractured vertebra and to reduce severe pain after the fracture. Bone cement such as Polymethylmethacrylate or calcium phosphate cement has been used for this procedure. However, serious neurological complication and pulmonary embolism might occur following the leakage of the cement.

In order to avoid these serious complications, it seems to be important to make an enough space in the vertebral body before injecting bone cement. For this purpose, we utilized balloon inflation and endoscopic resection of connective tissue for pseudoarthrosis following osteoporotic vertebral fracture. With this procedure, severe pain after the fracture improved dramatically. So far, we did not have any serious systemic complications

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はじめに

椎体形成術は1987年Galibertら[1]によって血管腫の治療に初めて行われた。その後転移性骨腫瘍[2)~4)]，血管腫，骨髄腫などの腫瘍[5]や骨粗鬆症性椎体骨折に対しても用いられるようになった。骨粗鬆症性椎体骨折に対しては，骨変形の矯正，予防と疼痛軽減の目的で用いられている。本稿では，骨粗鬆症性椎体骨折に対する椎体形成術の現状について解説する。

適　応

椎体形成術は，骨粗鬆症性椎体骨折後の偽関節が最もよい適応となる[6)7)]。偽関節の診断は①単純X線で椎体部にvacuum cleft像が存在するか，②前後屈像で椎体内に異常可動性が認められること，あるいは③磁気共鳴画像(MRI)において椎体内にT2強調像で高輝度性変化が見られることである。この場合に，椎体形成術は疼痛の軽減や日常生活動作(ADL)の改善に有効であるが，明らかな下肢神経症状を伴わないことが条件となる。

一方で，新鮮骨折例に対する椎体形成術も報告されている。中野ら[8]は，その適応として①神経麻痺を伴わない有痛性の骨粗鬆症胸腰椎骨折，②疼痛による離床が困難であり，その原因が明らかで画像診断と一致すること，③保存療法の予後不良因子の検討に基づいて，胸腰椎移行部の骨折で椎体後壁損傷を伴うもの，としている。この予後不良因子については未だ十分に検討されているとは言えず，今後の検討が待たれる。

使用する補填材料

現在骨折部の補填材料としては，Polymethylmethacrylate(PMMA)骨セメント，calcium phospate cement（CPC）あるいはハイドロキシアパタイトが用いられる。

PMMAは最大強度に達するまでの期間が短く，最大強度も約100 MPaと言われており，罹患椎体の強度を短期間で増加させる目的では最も適している。しかし，重合熱の問題や，モノマーの吸収による血圧低下問題が欠点としてあげられる。一方，CPCを使用した場合，骨伝導能があるために一部は骨に置換される可能性があることが利点である。しかし，その硬化には約3日間を要し，その間活動性の制限が余儀なくされることが欠点となる。また，両者について脊柱管内漏出や塞栓症の問題などが指摘されており，この点については骨セメント共通の欠点となる。

これらの欠点を補うために，松崎ら[9]はハイドロキシアパタイトを補填材料として使用している。ブロックを用いるために術後の矯正損失は少なく，術後4週間で10%の損失が見られた後は安定化する傾向にあったとしている。

Vertebroplastyとkyphoplasty

Vertebroplastyは，骨折した椎体の変形を矯正せずに椎体内にセメントを充填し，椎体の強度を高める手技である。一方，kyphoplastyは，圧壊した椎体の椎体高を復元するために椎体内にバルーンなどを挿入し，圧壊した椎体を矯正し，その結果生じた椎体内の空隙に骨セメントを注入する方法である[10)11)]。Kyphoplastyは造語であり，骨折部に形成された局所後弯を矯正する手技を併用した椎体形成術に対して用いられる。

合併症

椎体形成術に伴う合併症は，セメント注入に伴うものが多い。椎体へのセメント注入時の圧は2,000 kPaにも達し[12]，それに伴い椎体外への骨セメントのリークは20～71%に達すると言われている[13)~17)]。

このセメントの漏出は脊髄や神経根の圧迫の原因となり[18)19)]，下肢麻痺が続発することもある[20]。また椎体外静脈叢へのセメントの混入は肺塞栓症の原因となり[21)22)]，致死的な結果[23]を招くことも

ある。これら合併症のもととなるセメントの漏出は，セメント注入の器具や圧，粘調度によって左右される[24)~26)]。

筆者らの椎体形成術

上記合併症を考慮して，我々はバルーンと内視鏡下瘢痕切除を応用した椎体形成術を偽関節例に対して応用しているので，その概略を解説する。

1．手術手技

全身麻酔下(可能であれば低血圧麻酔下)に4点支持フレーム(hall frame)を使用し，腹臥位で行う。

(1) アプローチ

イメージ透視下に罹患椎の椎弓根を同定する。その背側部に左右それぞれ約2 cmの小皮切を加えた後，pedicle probe, tapを用いて経椎弓根的に椎体内に到達する。両側の椎弓根孔に連続性があることを確認するため椎弓根にユニバーサルカニューレを挿入し，片側から生理食塩水を注入し，反対側の孔からの流出を確認する。これが確認できれば，両側の椎弓根孔が椎体内の偽関節腔を通じて交通したことを示す。

(2) バルーン挿入・椎体矯正(図1)

片側より経椎弓根的に8 Frウロマチックバルーンを椎体内に挿入し，イソビスト®(一般名：イオトロラン)の注入によってバルーンを膨らませ，バルーンの位置，膨大状況，椎体高の増大をイメージ下に観察する。

(3) 内視鏡挿入・椎体内掻爬(図2)

バルーンを抜去して，膝関節鏡に用いる30度の斜視鏡を片側椎弓根から挿入し，対側椎弓根孔から鉗子を入れ，鏡視下に偽関節腔内肉芽組織を可及的に掻爬する。以上の操作を対側椎弓根からも行う。

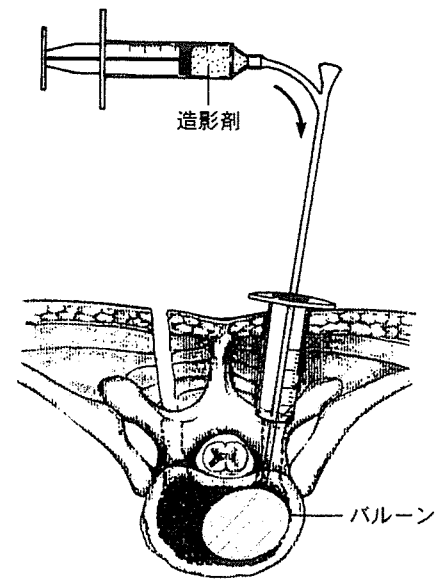

図1　バルーン挿入・椎体矯正

経椎弓根的にバルーンを椎体内に挿入し，造影剤(イソビスト®)でバルーンを膨らませる。この手技で椎体内に空隙が形成され，内視鏡による椎体内の観察が容易となる。

(文献27より)

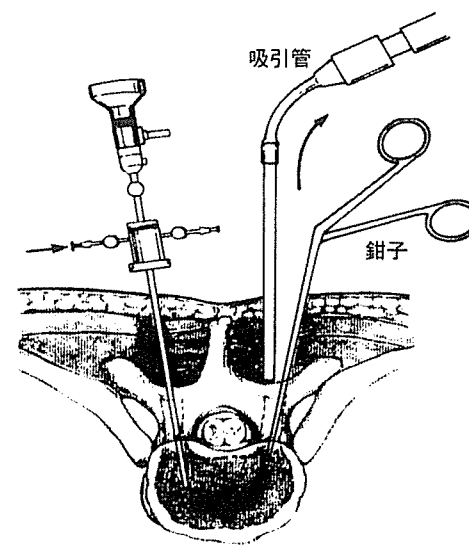

図2　内視鏡挿入・椎体内掻爬

一側椎弓根から30度斜視の関節鏡を挿入し，対側から曲がり鉗子を挿入して，偽関節部に形成された肉芽組織を可及的に掻爬する。

(文献27より)

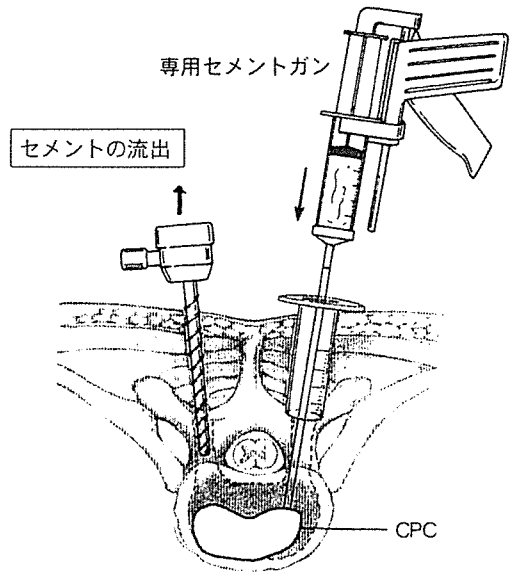

図３　椎体造影・CPC 注入

造影剤を椎体内に注入して椎体外への造影剤の漏れが無いことを確認した後，専用セメントガンを使用して calcium phosphate cement（CPC）を椎体内に注入する。

（文献 27 より）

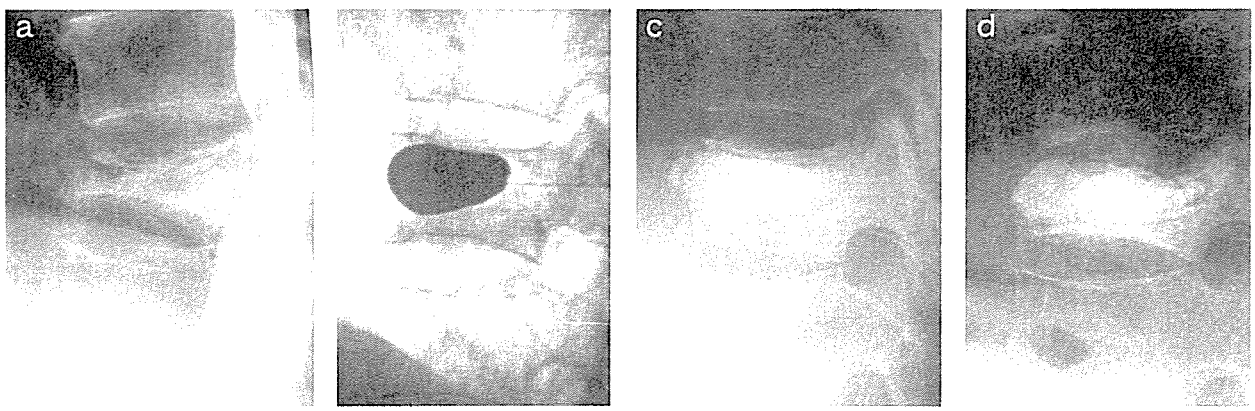

図４　77 歳男性，第 12 胸椎骨折の症例

77 歳男性，第 12 胸椎骨粗鬆症性椎体骨折後偽関節に対してバルーンによる椎体整復の後，内視鏡下に偽関節部の肉芽組織を掻爬し，calcium phosphate cement(CPC)を注入した。術直後椎体高が整復され，術後 1 年の時点で椎体高が約 80％に保持されている。

a．術前 myelography：椎体の圧壊と椎体内の vacuum cleft が確認できる。

b．術中透視画像：椎体内でバルーンが膨らむ様子が確認できる。

c．術直後 X 線像：椎体高は良く整復されている。

d．術後 1 年の X 線像：術直後に比較して約 80％の椎体高が保持されている。

（文献 27 より）