in 1988, and BMP were found to be a dimeric protein with a molecular weight of about 32,000 [15–17]. An important common feature of the BMP molecules is the position of cysteine residues in relation to the carboxyl terminus. The positions of these seven cysteine residues are the same as those in transforming growth factor (TGF)-β, indicating that BMP molecules are members of the TGF-β superfamily [18]. Today, the BMP family consists of about 15 BMP [19].

BMP include bone formation during embryogenesis, growth, and adulthood. In fracture healing, osteoprogenitor cells can respond to BMP and differentiate into osteoblasts. BMP bind to their receptors on progenitor cells, initiating signal transduction according to the following sequence. BMP molecules bind to a type IA or IB BMP receptor (BMPR-I) and to a type II BMP receptor (BMPR-II) to form a heterotetramer. These receptors are of the serine/threonine kinase type. As a result of BMP binding, BMPR-II phosphorylate the glycine/serine-rich domain of BMPR-I. BMPR-I then phosphorylate the C-terminal domain of Smads 1, 5, and 8. [Smads is a term identifying homologues of "Mothers against decapentaplegic" (Mad) and the related genes, Sma.] Smad 6 blocks the phosphorylation cascade by binding to BMPR-I. Following phosphorylation, Smads bind to Smad 4 and translocate to the nucleus. On the other hand, when Smads bind to Smad 6, the signal is terminated. Once inside the osteoblast nucleus, Smads initiate and activate Smad target gene transcription [20–22].

Among members of the BMP family, BMP-2, -4, and -7 possess a strong ability to induce bone formation. These BMP molecules have been synthesized successfully by DNA recombination techniques; the protein products (rBMP) have been shown to possess the bone-inducing effect of BMP [23,24]. Thus, human-type BMP (rhBMP) have become available for potential medical use. A number of preclinical studies have assessed the efficacy of rhBMP in healing of bone defects and acceleration of fracture healing.

2.2. Delivery systems for BMP

New bone formation in vivo cannot be obtained simply by injecting aqueous BMP solutions into the area where bone is needed. Delivery systems that retain BMP and release it slowly, as well as serving as scaffolding for new bone formation, are essential. A delivery system also must be biocompatible and biodegradable; lack immunogenicity, toxicity, and carcinogenicity; permit the biologic activity of BMP; be easily handled; be sterilizble; and be inexpensive to produce commercially. A large number of materials that satisfy these conditions have been considered as BMP delivery systems and tested in animals.

One of the first candidate materials was demineralized bone matrix (DBM), from which BMP were originally isolated [14,25]. Osteoconductive delivery systems have included collagenous materials, such as type I collagen (as sponges, gels, or fibrils) [19,26–30], and type IV collagen [31,32]; inorganic ceramic materials, such as hydroxyapatite (HA) (as a powder, granules, or blocks) [33,34], tricalcium phosphate (TCP) [35], glass ceramic, and other inorganic materials; cartilage- or bone-derived materials, such as coral, chitin, and bone mineral; and composites of different types of these materials [20]. BMP have also been used in combination with titanium and other metal alloys [36].

Among these candidates, the most effective material is type I collagen, which now is considered the "gold standard." Type I collagen, a biologically occurring polymer, is a major component of bone and a suitable scaffold. In addition, collagen is degraded and absorbed in vivo, allowing its disappearance after new bone is formed. Since this collagen was extracted from tendons and skin of pigs and cattle, an atelocollagen was developed from these sources largely eliminating antigenicity. In animal tests, many excellent results have been obtained using this collagen with BMP. This atelocollagen delivery system also is used for clinical trials of BMP but some antigenicity remains, posing a degree of risk of immunologic reaction when used repetitively or in large amounts. Furthermore, a potential risk exists for transmission of infectious disease [37–39]. Finally, biodegradability and other properties are difficult to adjust. To avoid these problems, synthetic degradable polymers have been examined as possible BMP delivery systems.

2.3. Synthetic polymers for BMP delivery system

Synthetic biodegradable polymers pose no danger of immunogenicity or possibility of disease trans-

mission. In addition, characteristics such as strength, degradability, and adhesiveness can be altered to facilitate clinical use.

Biodegradable polymers with high biocompatibility originated in the development of suture materials for surgery. These materials must be strong immediately after the operation when the tissue is sutured in vivo, but after the wound has healed, they ideally should degrade and be absorbed. For this purpose, many biodegradable polymeric suture materials with high biocompatibility have been developed, and large-scale screening tests were carried out. As a result, several kinds of synthetic polymeric suture material are now in clinical use. These include poly-α-hydroxy acids such as polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers (PLAGA). Their favorable characteristics as suture materials has prompted researchers to test their suitability as carriers for BMP.

BMP have been tested with a variety of biodegradable polymers including PLA, PGA, and PLAGA, and other polymers such as polyethylene glycol, poly-ε-caprolactone, and polyphosphazetes [40–46]. However, none has proven equal to collagen [47,48]. We therefore sought to develop new synthetic biodegradable polymers that would prove superior to collagen as BMP delivery system [49–52].

3. Development of new synthetic biodegradable polymers for rhBMP-2 delivery

3.1. Poly-D,L-lactic acid-polyethylene glycol block copolymer (PLA-PEG)

Among rhBMP, we tested rhBMP-2, which has been considered to possess greatest osteoinductive activity. First we tested biodegradable polymers for rhBMP-2 delivery that exhibited plasticity at room temperature. We synthesized PLA-PEG block copolymers of various molecular sizes with various PLA/PEG ratios (Fig. 1) [53,54]. Results were assessed in vivo by mixing each polymer with rhBMP-2 and implanting the mixture into the back muscles of mice for 3 weeks to determine its capacity to induce ectopic bone formation. The results showed superiority of a PLA-PEG block copolymer with a total molecular weight of approximately 9500 and a PLA/PEG molar ratio of approximately 3:2. Although this polymer worked well as a delivery system for rhBMP-2 and new ectopic bone was induced consistently, degradation of this polymer was somewhat slow; the material remained at the center of the rhBMP-2-induced ossicles.

PLA-PEG

$$\underbrace{H\text{-}(\text{-}O\text{-}CH(CH_3)\text{-}CO\text{-})_m}_{\text{PLA segment}}\text{-}O\text{-}\underbrace{(\text{-}CH_2\text{-}CH_2\text{-}O\text{-})_n}_{\text{PEG segment}}\text{-}\underbrace{(\text{-}OC\text{-}CH(CH_3)\text{-}O\text{-})_o\text{-}H}_{\text{PLA segment}}$$

PLA-DX-PEG

$$\underbrace{H\text{-}(\text{-}O\text{-}CH(CH_3)\text{-}CO\text{-})_m\text{-}(\text{-}O\text{-}CH_2\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CO\text{-})_n}_{\text{PLA-DX random polymer segment}}\text{-}O\text{-}\underbrace{(\text{-}CH_2\text{-}CH_2\text{-}O\text{-})_o}_{\text{PEG segment}}\text{--}$$

$$\text{--}\underbrace{(\text{-}OC\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}O\text{-})_p\text{-}(\text{-}OC\text{-}CH(CH_3)\text{-}O\text{-})_q\text{-}H}_{\text{PLA-DX random polymer segment}}$$

Fig. 1. Structural formulas of poly-D,L-lactic acid-polyethylene glycol block copolymer (PLA-PEG) and poly-D,L-lactic acid-p-dioxanone-polyethylene glycol block copolymer (PLA-DX-PEG). Both are A–B–A type polymers. m, n, o, p, q: number of units.

3.2. Poly-D,L-lactic acid-para-dioxanone-polyethylene glycol block copolymer (PLA-DX-PEG)

To optimize degradation of the polymer used to deliver rhBMP-2, para-dioxanone molecules were randomly inserted into the PLA segments of the PLA-PEG polymer without changing the total molecular weight [55]. This PLA-DX-PEG block copolymer represents a novel material (Fig. 1). Use of this new polymer as a delivery system for rhBMP-2 resulted in complete replacement of the implants by new bone with no visible remnants of the polymer, presumably reflecting a favorable degradation rate. In vivo comparison showed that the rhBMP-2/PLA-DX-PEG composite implants could induce new bone formation more effectively than a PLA-PEG/rhBMP-2 composite.

PLA-DX-PEG consists of a copolymer of polylactic acid and para-dioxanone and a homopolymer of polyethylene glycol. Individually, these polymers already have been used clinically as suture materials, screws, and delivery systems for other drugs. Therefore, PLA-DX-PEG is anticipated to be safe for clinical use as well. Nevertheless, further tests in large animals or primates are essential before this bone-inducing implant can be studied in a clinical setting. At room temperature, PLA-DX-PEG with molecular weight of 9500 is a firm gel that is easy to manipulate (Fig. 2). We tested whether this novel polymer could act as an effective rhBMP-2 delivery system.

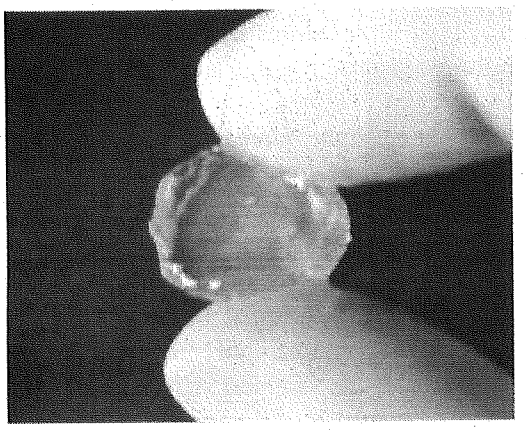

Fig. 2. Appearance of PLA-DX-PEG (reprinted from [51], with permission from Marcel Dekker Inc.) requires copyright permission since we previously published in Tissue engineering and novel delivery systems [51]. At room temperature, PLA-DX-PEG with a molecular weight of 9500 is a firm gel, which can be shaped and is easy to manipulate.

The PLA-DX-PEG polymer mass was dissolved in organic solvent (acetone) and mixed with rhBMP-2 solution. After agitation, acetone was removed by evaporation with a centrifuge evaporator to return the polymer to its native state (Fig. 3). Male ddy mice (5 weeks old) were anesthetized with diethyl ether, and test implants were aseptically placed into the left dorsal muscle pouches (one per animal). Three weeks after surgery, the implants were harvested together with surrounding tissues. Soft X-ray radiographs and histologic examination of ectopic new bone showed mature trabecular bone and hematopoietic bone marrow (Fig. 4). No evidence of inflammatory or foreign-body reaction from the host could be found in tissues adjacent to the new bone.

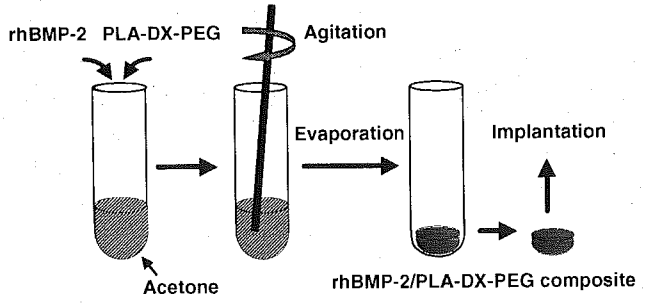

Fig. 3. Methods for combining PLA-DX-PEG polymer with rhBMP-2. PLA-DX-PEG was dissolved in organic solvent, and rhBMP-2 solution was mixed in it. Evaporation with a centrifuge evaporator removed the solvent, so an rhBMP-2/PLA-DX-PEG composite implant was obtained.

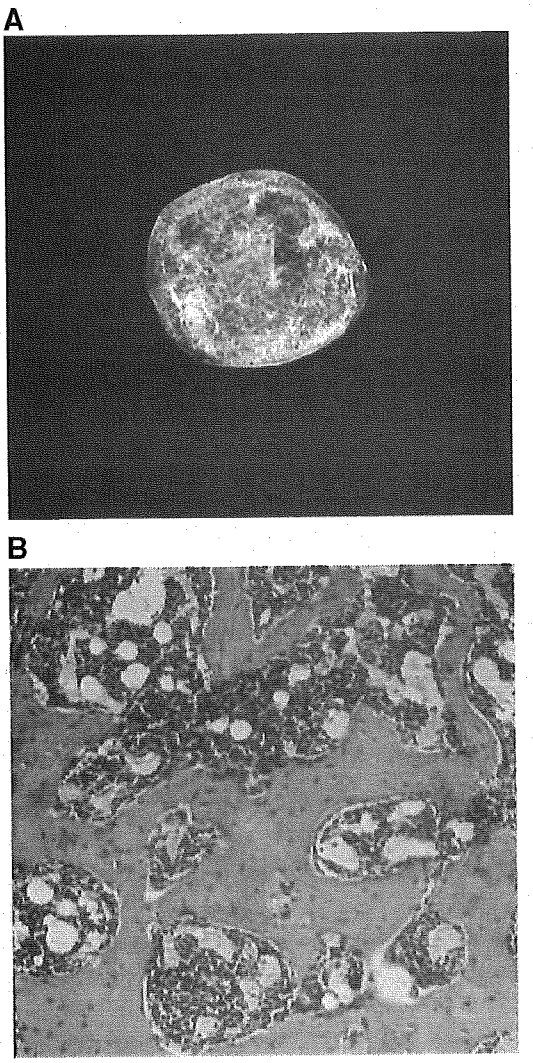

Fig. 4. Ectopic bone formation induced by the rhBMP-2/PLA-DX-PEG composite (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. The composite was placed in the back muscle of mouse, which was harvested after 3 weeks. Ectopic new bone showed mature bone trabeculae with hematopoietic bone marrow. (A) Soft X-ray radiograph. (B) Photomicrograph. Hematoxylin and eosin stain.

We compared the PLA-DX-PEG polymer and collagen as rhBMP-2 delivery systems. For a positive control implant, an aliquot of rhBMP-2 solution was absorbed by a type I collagen sponge disc, lyophilized, and compressed to form an implant of the same volume as the PLA-DX-PEG implant. As a result, implants containing more than 0.5 μg of rhBMP-2 showed bone formation in both groups. Therefore, in terms of ability to elicit new bone formation by rhBMP-2, the PLA-DX-PEG delivery system was

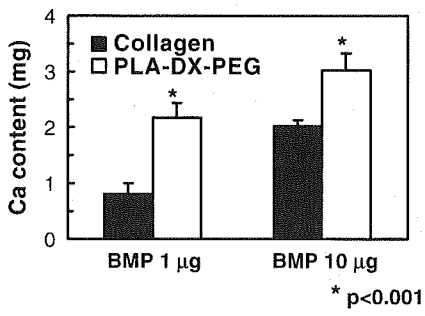

Fig. 5. Comparison of calcium content of new bone obtained using an PLA-DX-PEG system and a collagen system (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. The calcium content of new bone obtained using the PLA-DX-PEG system was significantly higher than that obtained using the collagen system for both 1 μg and 10 μg of rhBMP-2.

equal to the collagen system. Amounts of calcium in the new bones induced in the PLA-DX-PEG and the collagen delivery system groups with 1 μg or 10 μg of rhBMP-2 were quantified. The mean calcium content in ossicles from the PLA-DX-PEG group was significantly higher than in those from the collagen group at both doses (Fig. 5). Therefore, this polymeric delivery system may permit reduction of the effective dose of rhBMP-2 for clinical use compared to doses used with collagen.

4. Repair of bone tissues using rhBMP-2 and new synthetic polymers

4.1. Repair of bone defect using composites of rhBMP-2 and synthetic polymers

Use of rhBMP-2 in combination with a delivery system material ideally forms new bone of the same shape as that of the original bone. Development of synthetic biodegradable polymers is believed to permit control of the size and shape of newly formed bone if an appropriate delivery system is used. For this purpose, the hard gel type of PLA-DX-PEG is suitable [55].

To test whether this novel polymer functions appropriately in large bone defects in vivo, we implanted rhBMP-2/PLA-DX-PEG composites in rat iliac bone defects 4 mm in diameters, which is considered a critical size for informative testing. We examined these defects using radiographic and histologic methods. The bone defect was repaired in a manner showing rhBMP-2 dose dependence and time dependence. Histologic analysis of the specimens revealed that defects treated with 10 μg of rhBMP-2 were filled with dense trabecular bone with no evidence of polymer remnants at 4 weeks postoperatively. At the host–defect interface, new bone had formed adjacent to the host bone (Fig. 6). These

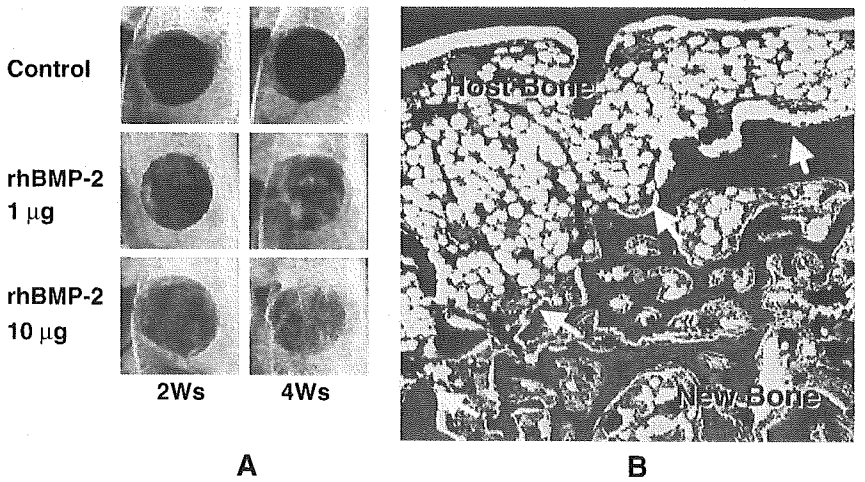

Fig. 6. Repair of a bone defect using PLA-DX-PEG as a delivery system for rhBMP-2 (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. A cylindrical defect 4 mm in diameter was created in the ilium of rats, and was filled with rhBMP-2/PLA-DX-PEG composite. (A) The defect was repaired with newly formed bone in a manner dependent on rhBMP-2 dose and on time. (B) New bone with hematopoietic marrow and bony trabeculae was formed adjacent to the host bone (arrows). Hematoxylin and eosin stain.

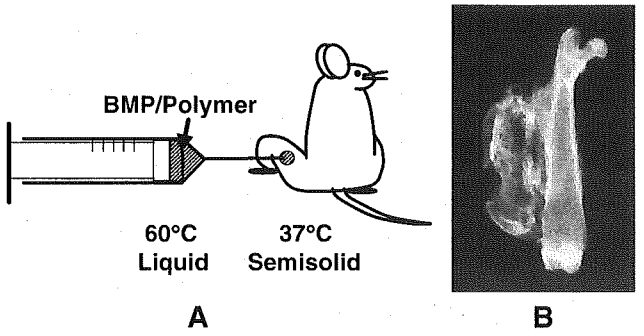

Fig. 7. Injectable polymeric delivery system for rhBMP-2 (reprinted from [56], with permission from Elsevier) requires copyright permission since we previously published in Bone [56]. (A) When heated to 60 °C, the rhBMP-2/PLA-DX-PEG composite can be injected percutaneously, avoiding need for surgical implantation. Subsequently, implants become firm upon cooling to body temperature, resulting in semisolid polymeric implants in vivo. (B) Soft X-ray radiograph of new orthotopic bone formed by injection of the rhBMP-2/PLA-DX-PEG composite in the muscle pouch on the abraded surface of the murine femur 3 weeks after injection.

results suggest that rhBMP-2 in the PLA-DX-PEG polymer delivery system should be suitable for eliciting bone formation and healing in large bone defects.

4.2. Injectable polymeric delivery systems for rhBMP-2

Injectable delivery systems for rhBMP-2 could provide a less invasive method for repair of bone defects, avoiding extensive invasive surgery [56]. Clinical indications might include fresh fractures, nonunion, or delayed union of bone causing serious difficulty in fracture treatment, as well as large defects often associated with bone tumor resection. As far as we know, no such delivery system has been developed or reported.

The new synthetic biodegradable PLA-DX-PEG polymers feature an exquisite temperature-dependent liquid–semisolid transition and work well as an injectable rhBMP-2 delivery system. The thermosensitive property of the rhBMP-2/PLA-DX-PEG composite permits percutaneous injection after heating. Fluidity of the composite decreases as it cools to body temperature, and the resultant semisolid form provides a scaffold for bone formation as it gradually releases rhBMP-2 into its immediate surroundings.

The rhBMP-2 molecule is a heat-stable protein [57]. For example, biologic activity of rhBMP-2 was unchanged after heating to 60 °C for 30 min. Considering the heat-stable character of rhBMP-2, PLA-DX-PEG with molecular weight of 6400 could be a suitable system for injectable delivery of rhBMP-2. Together with rhBMP-2, this polymer heated to 60 °C could be injected as a liquid and then turn to a semisolid form in vivo at 37 °C. The properties of the polymer would allow retention of BMP for a period of time sufficient to elicit new bone formation while serving as a scaffold for further bone growth. Eventually, it would be completely replaced by new bone, avoiding surgery for removal since the polymer is biodegradable (Fig. 7A). To further demonstrate the efficacy of this polymer, 25 mg of PLA-DX-PEG mixed with 10 μg of rhBMP-2 was heated at 60 °C for 5 min and injected using a 14-gauge needle into muscle overlying the surface of the murine femur. Three weeks after injection, new bone was found at the injection site, and was attached to the surface of the femur (Fig. 7B). This new type of injectable osteoinductive material should allow less invasive surgery involving restoration or repair of bone.

We also tested this injection technique in spinal fusion [58]. The rhBMP-2/PLA-DX-PEG composites were injected into the anterior longitudinal ligaments

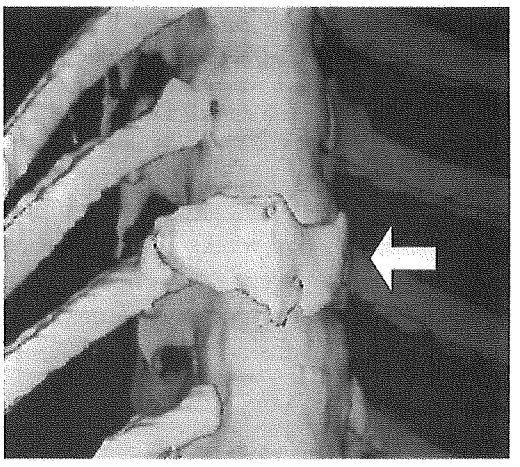

Fig. 8. Spinal fusion by injection of the rhBMP-2/PLA-DX-PEG composite (reprinted from [58], with permission from Lippincott Williams and Wilkins) requires copyright permission since we previously published in J. Spinal Disord. [58]. PLA-DX-PEG with rhBMP-2 was injected into the anterior longitudinal ligament of the spine in dogs. New bone was formed on the anterior aspects of vertebrae after 6 weeks (3D-CT, arrow).

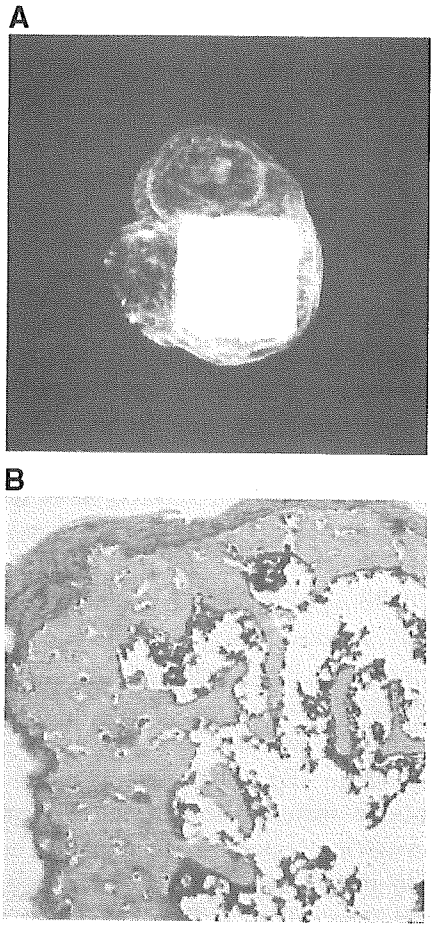

Fig. 9. Ectopic bone formation by hydroxyapatite (HA) with the rhBMP-2/PLA-DX-PEG (reprinted from [54], with permission from The Journal of Bone and Joint Surgery, Inc.) requires copyright permission since we previously published in J. Bone Joint Surg. Am. [54]. The rhBMP-2/PLA-DX-PEG composite was placed in the pores the HA block, which was inserted in the back muscle of mice. (A) Soft X-ray radiograph showed the new bone surrounding HA at 3 weeks. (B) Histologic examination also showed new bone within the pores of the HA.

of the canine spine. Six weeks later, new bone had formed, bridging between the vertebrae anteriorly (Fig. 8). If a pneumoscopic technique were used jointly, anterior spinal fusion might be accomplished by a less invasive approach.

4.3. Combination of the rhBMP-2/polymer composites with other materials

The hydrophilic nature of the PLA-DX-PEG polymer causes it to swell on contact with water. This physical property provides an additional advantage for use of the polymer in combination with porous materials. When a solid implant with pores filled with the rhBMP-2/PLA-DX-PEG composite is implanted, the composite will swell, extruding itself from the pores to form a layer of composite.

To test this property, a combination of the rhBMP-2/PLA-DX-PEG composite with porous hydroxyapatite (HA) was used [54]. The rhBMP-2/PLA-DX-PEG composite was placed in the pores of an HA block, which then was inserted into the back muscle of mice. Over 3 weeks, new bone had formed to surround the HA. Histologic examination showed new bone formation within the pores of the HA as well (Fig. 9).

Next, rhBMP-2 (120 μg) was mixed with the polymer (120 mg) and impregnated into titanium fiber-mesh cylinders [59]. Three 5-mm cylinders were placed end-to-end to fill a 15-mm defect created in the humerus of adult rabbits and stabilized with an intramedullary rod. In controls, the titanium fiber-mesh cylinders contained the polymer but not rhBMP-2. Six weeks after implantation, new bone had formed on the surface of the implant and had bridged the

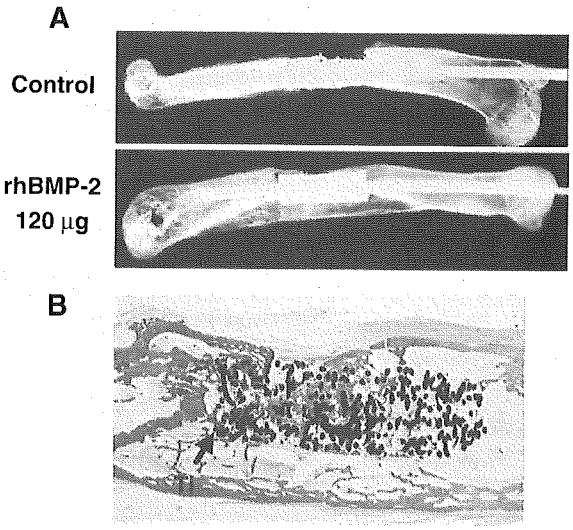

Fig. 10. Repair of a bone defect with a titanium syringe implant with the rhBMP-2/PLA-DX-PEG composite (reprinted from [59], with permission from 6 Wiley) requires copyright permission since we previously published in J. Biomed. Mater. Res. [59]. A bone defect of 1.5 cm was created in the humerus of rabbits, and three 5-mm implants were placed in it. These were stabilized with an intramedullary rod. (A) While the bone defect was not repaired in control groups, the defect was restored in the 120 μg rhBMP-2 group after 5 weeks. (B) Histologic examination showed that new bone also had formed within the titanium mesh (Ti), and that new bone had formed adjacent to the host bone. Hematoxylin and eosin stain.

defect. Defects treated with control implants were not repaired (Fig. 10). These results provide strong evidence that composite implants using rhBMP-2, synthetic degradable polymers, and compatible materials provide enhanced regenerative potential for the repair of a large bone defect. These techniques can repair bones whose function requires great strength, as a combination of rhBMP-2/PLA-DX-PEG composite with HA or titanium represents a mechanically durable osteoinductive material.

4.4. Development of a new artificial joint that restores a bone defect

Total hip arthroplasty (THA) has become essentially the standard procedure for treatment of various hip lesions. However, one limitation of this operation has been the eventual loosening of the prosthesis from periprosthetic bone loss. At revision surgery, various degrees of bone defect, both in the proximal femur and the acetabulum, often are encountered; these present challenges for sufficiently solid fixation of a new prosthesis. Alternative approaches aimed at overcoming this problem have included special design of the revision prosthesis and allo- or autogeneic bone grafting in combination with or without materials such as hydroxyapatite. If such bone loss can be repaired with use of rhBMP-2, revision surgery might be made more effective.

To address the problem of loosening of the prosthesis, we developed a new prosthesis combined with rhBMP-2/PLA-DX-PEG composite [60]. We tested efficacy of the rhBMP-2-containing prosthesis in reconstructing a bone defect in a canine model where the medial half of the proximal femur was resected to create a defect that was repaired with rhBMP-2/PLA-DX-PEG composite. Twelve weeks after implantation, the original bone defects in the rhBMP-2 treatment groups showed repair (Fig. 11). Thus, this type of hybrid prosthesis may represent a new modality for repair of bone defects or restoration of lost bone mass encountered in revision arthroplasty.

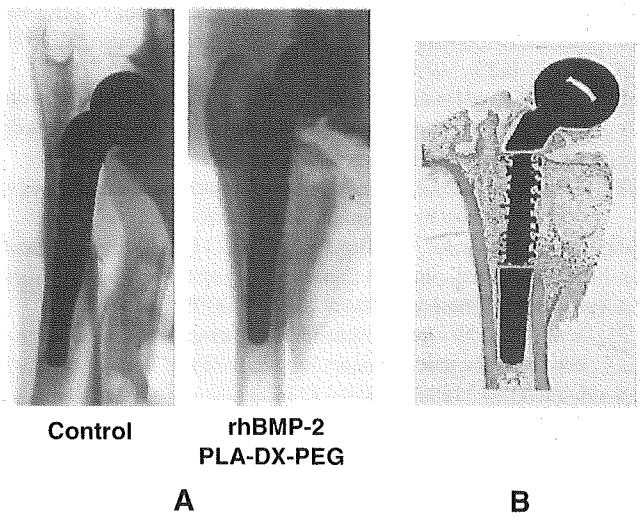

Fig. 11. Repair of a periprosthetic bone defect using PLA-DX-PEG/rhBMP-2 composite adherent to the prosthesis (reprinted from [60], with permission from Elsevier) requires copyright permission since we previously published in Biomaterials [60]. (A) Twelve weeks after implantation, the implant with rhBMP-2/PLA-DX-PEG showed new bone formation at the defect site. In the control group without rhBMP-2, only a scant amount of new bone was seen at the cut ends of the defects, which were not repaired. (B) By microscopic examination of sections in the rhBMP-2 treatment group, the new bone on the surface of implants showed normal histology with hematopoietic marrow and bony trabeculae. New bone formation also was observed within the pores of the titanium mesh. Hematoxylin and eosin stain.

5. Conclusions

A new delivery system using PLA-DX-PEG enabled creation of various osteoinductive materials that could be used to heal fractures and repair large bone defects. Importantly, this new rhBMP-2 delivery system was developed using synthetic biodegradable polymers, avoiding potential risks of disease transmission or immunogenicity associated with use of animal collagen or allogeneic bone grafts. Moreover, this system avoids problems of autogenous bone grafts such as limited supply of donor bone and the need for additional surgery to harvest the bone, with the risk of additional morbidity.

In summary, this new rhBMP-2 delivery system represents an innovative potential therapy that is safe, efficacious, and less invasive than current approaches for repair of damaged bone. Further work will be necessary to determine whether the biocompatible and biodegradable properties exhibited by the PLA-DX-PEG polymers in these studies are replicated during the practical application of rhBMP-2 in patient care.

Acknowledgements

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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.

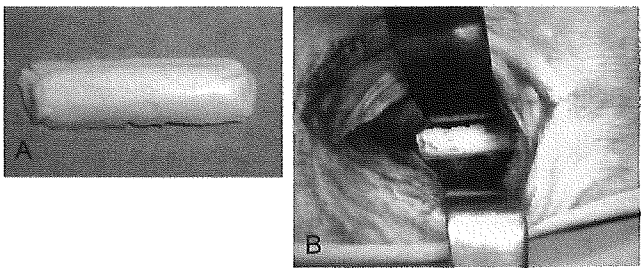

Figure 2. **A**, Prepared β-TCP dough implant. **B**, β-TCP dough was placed on the L5–L6 intertransverse region.

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-

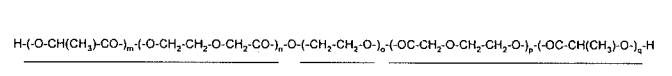

Figure 1. Structural formula of PLA-DX-PEG. The subscripts m, n, o, p, q represent variable number of units.

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.

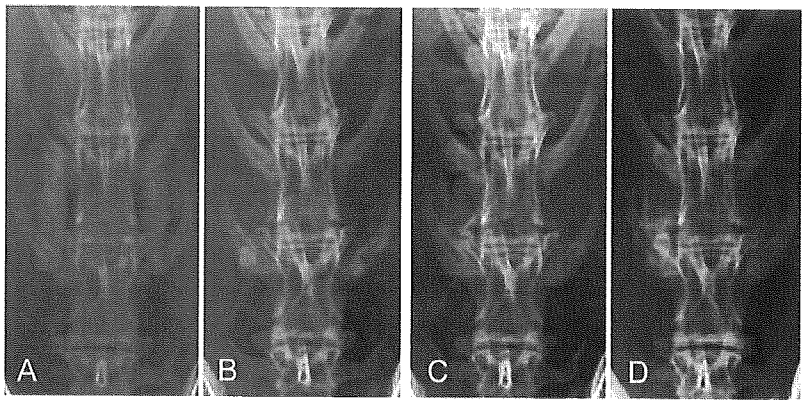

Figure 3. Representative posteroanterior radiographs of rabbit spines in the BMP 30 group, immediately after surgery (**A**), at 2 weeks (**B**), at 4 weeks (**C**), and at 6 weeks (**D**). In the radiograph, a remarkable fusion mass is visible between the L5–L6 transverse process at 6 weeks.

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

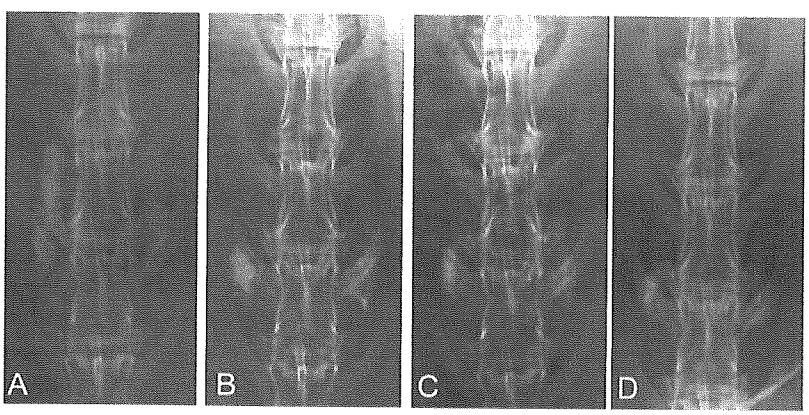

Figure 4. Representative posteroanterior radiographs of rabbit spines in the BMP 0 group, immediately after surgery (**A**), at 2 weeks (**B**), at 4 weeks (**C**), and at 6 weeks (**D**). In the radiograph, no fusion mass between the L5–L6 transverse process is visible.

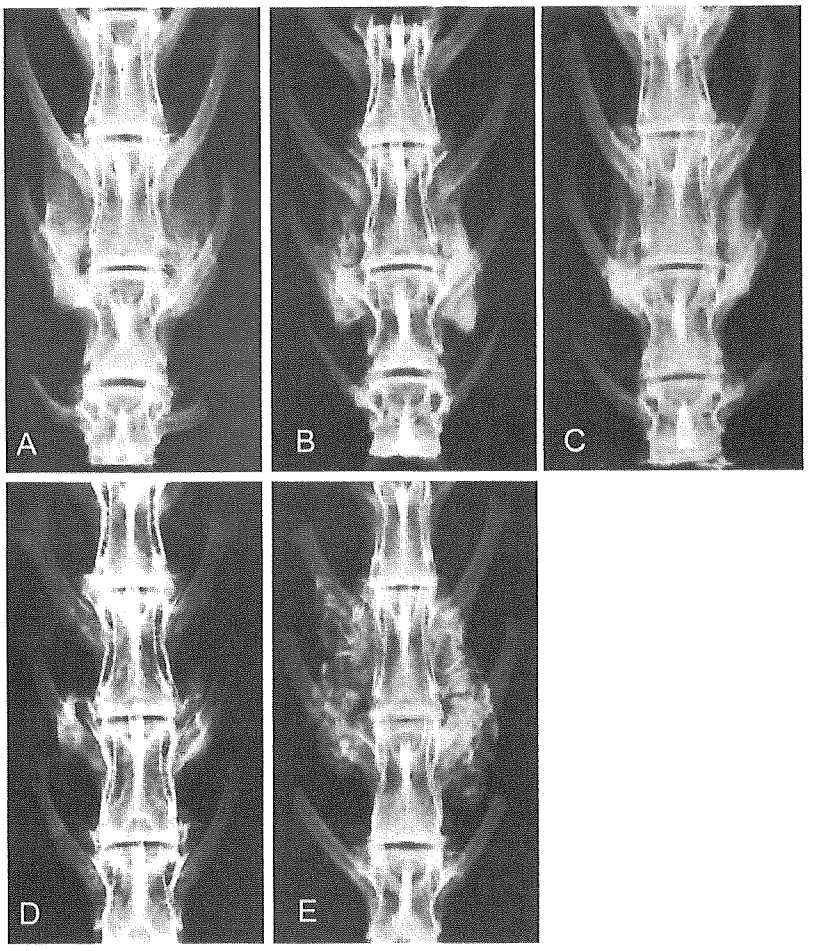

Figure 5. Harvested lumbar spine 6 weeks after surgery. BMP30 (**A**), BMP15 (**B**), BMP7.5 (**C**), BMP0 (**D**), autogenous bone (**E**).

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.

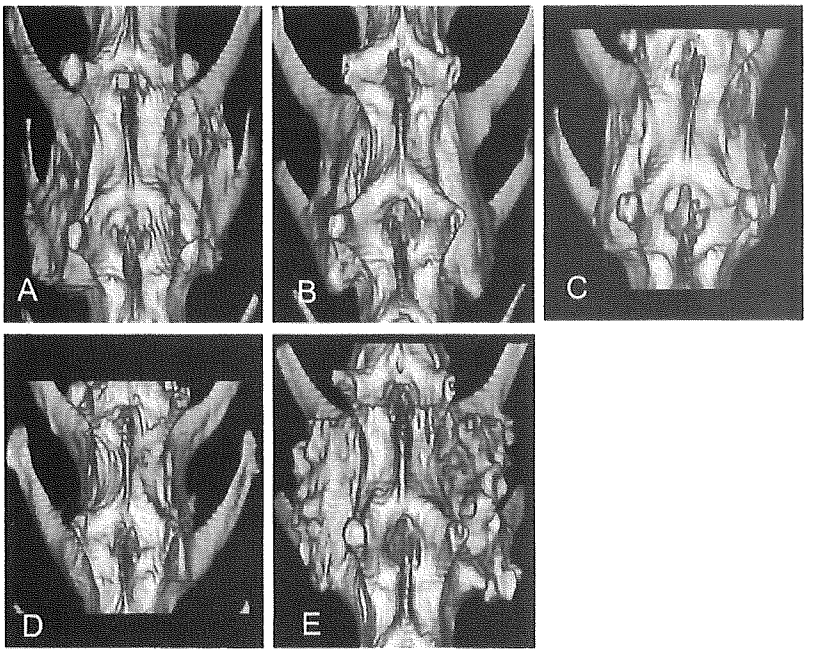

Figure 6. Three-dimensional-CT at 6 weeks after surgery. BMP30 (**A**), BMP15 (**B**), BMP7.5 (**C**), BMP0 (**D**), autogenous bone (**E**).

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

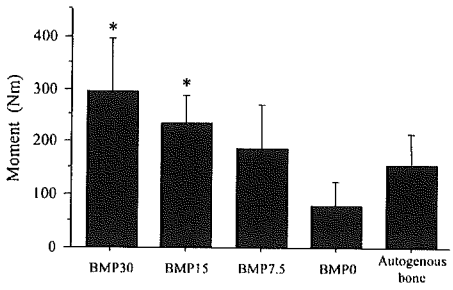

Figure 7. Results of three-point flexion bending tests for each specimen. These results indicate the fusion sites of BMP 30 and BMP 15 groups are stiffer than BMP 0 group. *, statistically significant difference from BMP 0 group.

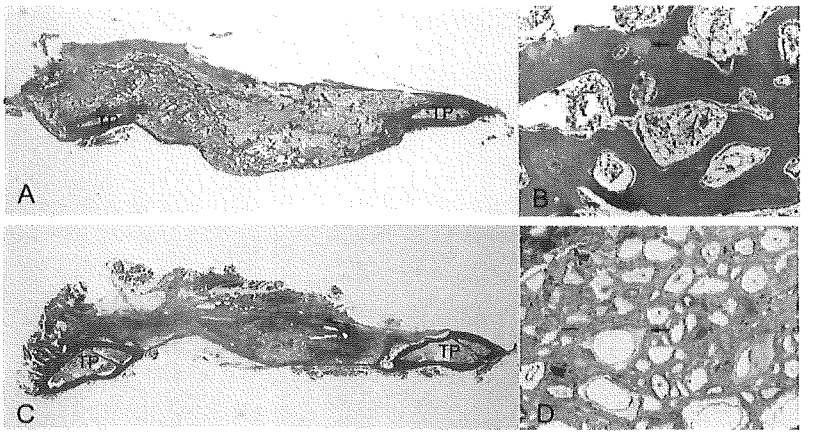

Figure 8. Hematoxylin and eosin stained sagittal section of L5–L6 intertransverse region in BMP 15 (**A,B**) and BMP 0 (**C,D**) group. New bone formation between the L5–L6 transverse process (TP), the cortical rim around fusion mass, and tiny residual β-TCP (arrow) was seen in BMP 15 group. The specimen 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.

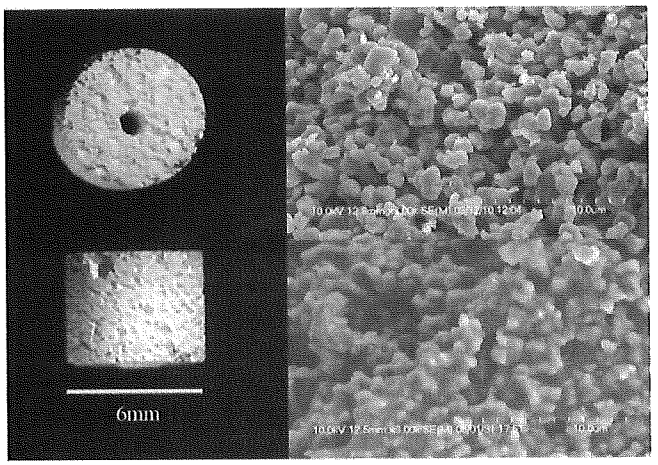

Fig. 1. Macroscopic aspect of β-TCP cylinders: images at left are axial and side views of the β-TCP cylinder, and at right are SEM micrographs of the β-TCP cylinder coated with PLA-DX-PEG ×3000 (above) and the noncoated control (×3000, below). The β-TCP cylinder consists of 1 μm sintered β-TCP granules 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