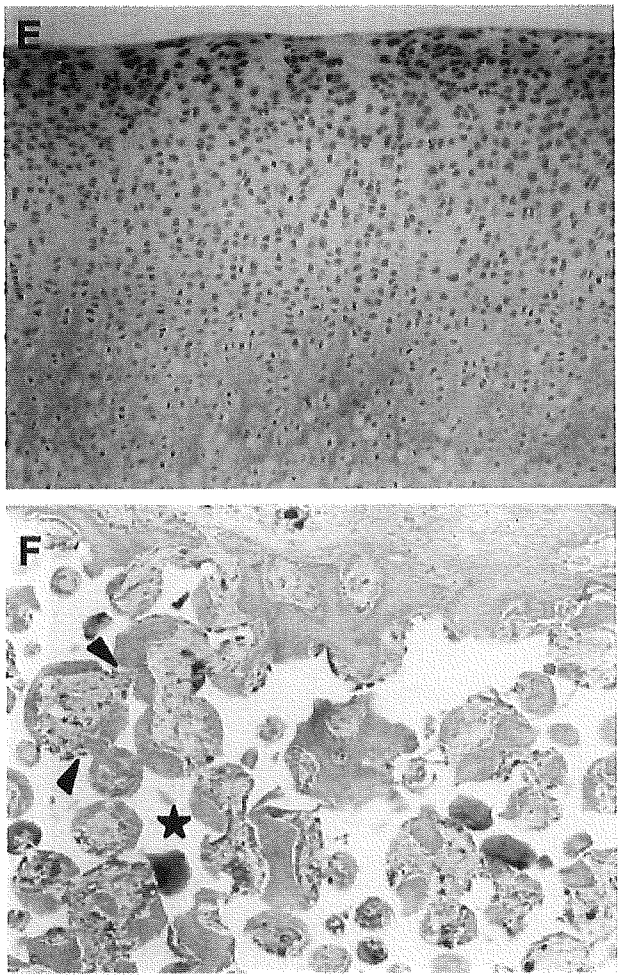

Fig. 5. (*continued*).

microscopically so well integrated with the surrounding host cartilage, the histological analysis revealed a slight discrepancy between safranin-O staining and immunoreactivity for type-II collagen [Fig. 6(J, L)]. This may be accounted for by the fact that chondrocytes near the junction with host cartilage produced less matrix than did those located more centrally within the regenerated tissue, where staining with safranin-O was absent only from the superficial zone, as in normal articular cartilage.

Cbfa1, a member of the Runt-domain family of transcriptional factors, is expressed not only in all osteoblasts, but also in chondrocytes and in earlier prechondrogenic mesenchymal condensations[47–49]. Furthermore, Cbfa1 is known to play an essential role in the differentiation not only of osteoblasts but also of chondrocytes, both at an early and a later stage of the process[50,51]. CD105 is a putative cell-surface marker for MSCs, which have the ability to undergo chondrogenesis, osteogenesis and adipogenesis[52,53]. One week after implantation, numerous cuboidal osteoblasts migrated into the pores of the IP-CHA scaffold from the host bone marrow (Fig. 3). And within peripheral pores, they had already begun to form bone tissue. The subchondral space above the IP-CHA scaffold was filled with an agglomeration of rounded fibroblast-like cells, which registered positive for Cbfa1 and/or CD105. They appeared to have migrated from the adjacent bone marrow, either directly, or indirectly via the interconnecting pores of the IP-CHA. These findings suggest that the aggregating fibroblast-like cells might have the potential for chondro/osteogenesis.

According to our findings, one of the keys to successful articular cartilage regeneration might be the activation of a subchondral repair process, thereby enabling chondroblastic/osteoblastic cells to effectively aggregate within the subchondral space. In rabbits, small, 3-mm-diameter, full-thickness articular cartilage defects heal spontaneously with repair tissue, which is composed of hyaline-like or fibrous cartilage. In adolescent rabbits (approximately 3 months old), osteochondral defects repair better and more rapidly than do those in adults[34,54]. Furthermore, adolescent rabbits have a larger population of metabolically active bone-marrow MSCs. Hence, in the present study, we established a large (4-mm-diameter) full-thickness defect model, it being necessary to exceed the upper limit (3 mm in diameter) for spontaneous repair. And since our system involved no cell-expansion step *in vitro*, the adolescent (rather than the adult) rabbit model was considered to be advantageous in its possession of a larger population of metabolically active bone-marrow MSCs.

A basic requirement for biomaterials is that they be non-carcinogenic and elicit no inflammatory reaction due to cytotoxicity or immunogenicity[55]. The BMP/PLA–PEG/IP-CHA composite is believed to meet these criteria. The PLA and PEG homopolymers and hydroxyapatite have been shown to be compatible and safe for clinical applications[30,56]. In addition to these safety features, it is crucial that biomaterials are easy to handle in clinical settings.

Table III
Results of the histological scoring

Group	No. of defects	Cell morphology	Matrix-staining	Structural integrity	Surface regularity	Thickness	Reconstruction of subchondral bone	Integration with adjacent cartilage	Total score
Group I: 3 weeks	6	3.6 ± 0.5*	2.0 ± 0	1.3 ± 0.5	2.0 ± 0.9	1.2 ± 0.4	0.8 ± 0.7	1.8 ± 0.4§	12.8 ± 2.4*
Group II: 3 weeks	6	1.7 ± 0.5	1.0 ± 0.6	0.5 ± 0.5	0.5 ± 0.5	0.7 ± 0.5	0.5 ± 0.4	1.0 ± 0.6§	5.8 ± 2.6
Group III: 3 weeks	6	2.2 ± 0.8	1.3 ± 0.5	0.5 ± 0.5	0.7 ± 0.4	1.0 ± 0	0.5 ± 0.4	1.2 ± 0.8§	7.2 ± 2.2
Group IV: 3 weeks	6	1.3 ± 0.5	0.8 ± 0.4	0.7 ± 0.5	1.3 ± 0.5	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	5.1 ± 1.1
Group I: 6 weeks	6	3.8 ± 0.4*	2.3 ± 0.5*	1.5 ± 0.5§	2.2 ± 1.0	1.2 ± 0.4§	1.8 ± 0.4§	1.5 ± 0.5	15.0 ± 2.1*
Group II: 6 weeks	6	1.8 ± 0.4	1.5 ± 0.5	1.2 ± 0.4	1.5 ± 0.5	1.2 ± 0.4	1.3 ± 0.5§	1.2 ± 0.4	9.7 ± 1.2
Group III: 6 weeks	6	1.7 ± 1.0	1.0 ± 0.8	0.8 ± 0.4	1.7 ± 0.5	0.7 ± 0.5	1.3 ± 0.5§	1.2 ± 0.4	8.3 ± 3.0
Group IV: 6 weeks	6	1.5 ± 0.5	0.5 ± 0.4	0.5 ± 0.5	1.3 ± 0.5	0.7 ± 0.5	0.5 ± 0.3	0.8 ± 0.4	5.7 ± 1.9

Values represent the average score ± SD for each category. *$P < 0.01$ vs groups II, III, and IV. §$P < 0.01$ vs group IV.

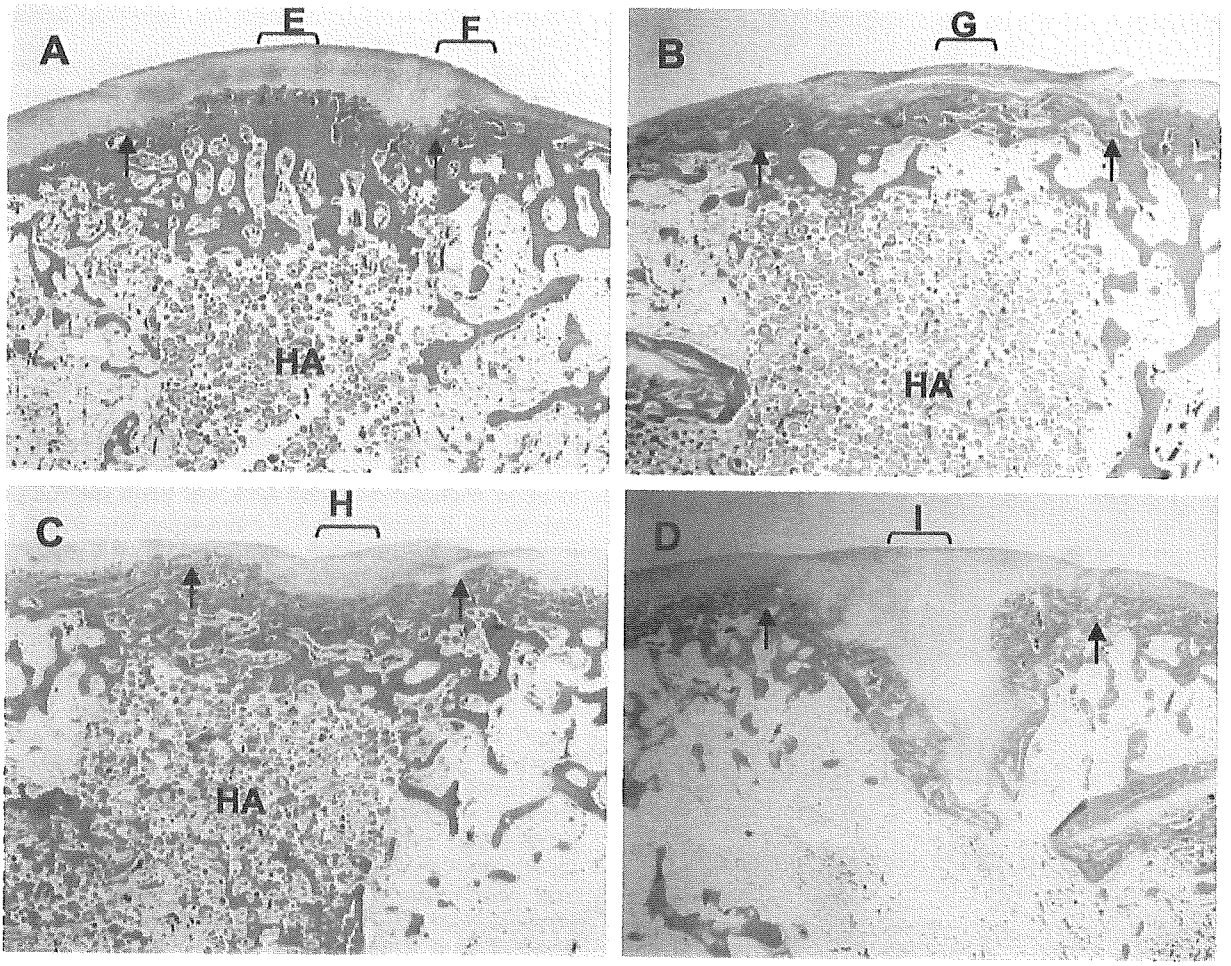

Fig. 6. Histological photomicrographs of defects 6 weeks after implantation with either the BMP/PLA–PEG/IP-CHA composite [(group I) A, E, F, J–L], the PLA–PEG/IP-CHA composite [(group II) B, G], or IP-CHA alone [(group III) C, H], and in the absence of treatment [empty (group IV) D, I]. Arrows indicate the margins of the defect. HA represents the implanted IP-CHA scaffold. Highly magnified images of the regions indicated in (A–D) are represented in (E–I). (A) The defect treated with the BMP/PLA–PEG/IP-CHA composite (group I) was filled with regenerated subchondral bone, which also penetrated the pores of the implant. The subchondral bone was covered with a layer of regenerated cartilage tissue of almost normal thickness. (B, C) In the control groups (group II and III), the defects were filled with a hypercellular type of fibrous tissue with regeneration of subchondral bone. The surface the repaired tissue was rough. (D) Without treatment (group IV), the defect site was predominantly replaced by thick fibrocartilage tissue with a thin layer of irregular subchondral bone. (E) The central region of the regenerated articular cartilage layer (group I). The repaired tissue has a hyaline-like appearance and is undergoing organization into vertical columns. The four horizontal strata characteristic of normal articular cartilage are apparent. (F) The junction between host and regenerated cartilage is continuous, and very little fibrillation of the articular surface is apparent (group I). (G–I) The repaired tissue is mainly of a fibrous nature (group II–IV). (J–L) Safranin-O staining (J), and immunostaining for type-I collagen (K) and type-II collagen (L) at the junction between host and the regenerated cartilage (group I). Magnification: (A–D) = 10×; (E–I) = 100×; (J–L) = 40×. (A–I): H&E staining.

Current techniques using cultured chondrocyte suspensions or collagen gels are complicated by problems associated with cell retention. Our composite material circumvents these problems. Furthermore, our material may be shaped into a "ready-to-use" form. It is possible to adjust its size and shape to suit the dimensions of the defect prior to implantation.

In conclusion, we have successfully induced the repair of articular cartilage defects within a relatively short period of time by combining rhBMP-2 with two biomaterials: IP-CHA as a scaffold and PLA–PEG as a carrier for rhBMP-2. The BMP/PLA–PEG/IP-CHA composite represents a new and promising technology for the engineering of articular cartilage. Clinical applications for the treatment of both osteoarthritis and articular cartilage injuries are also anticipated. Further studies involving long-term observations in both adolescent and adult animals are currently underway.

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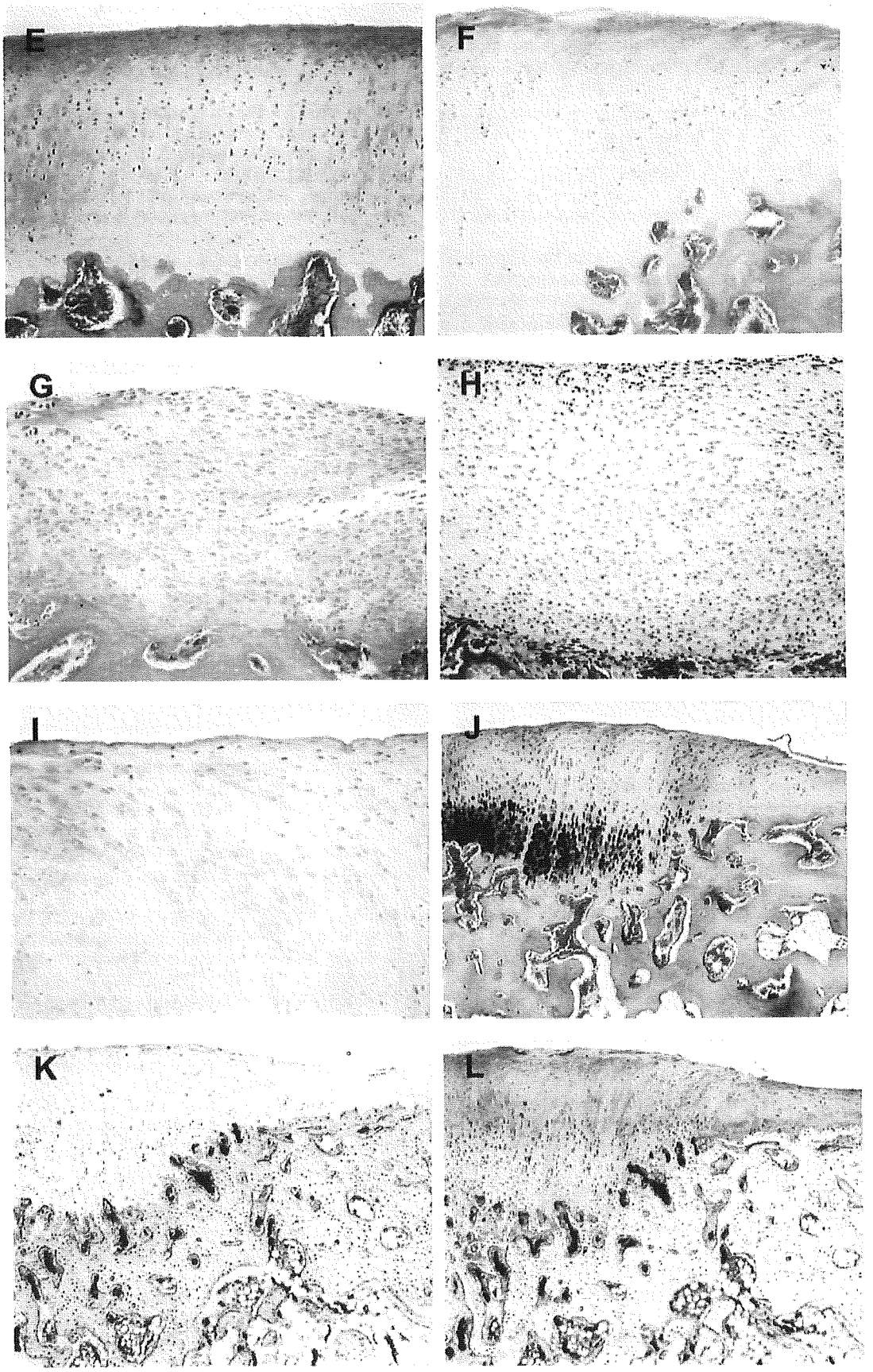

Fig. 6. (*continued*).

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Synthetic biodegradable polymers as drug delivery systems for bone morphogenetic proteins

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Abstract

Bone morphogenetic proteins (BMP) induce bone formation in vivo, and clinical application in repair of bone fractures and defects is expected. However, appropriate systems to deliver BMP for clinical use need to be developed. We synthesized a new synthetic biodegradable polymer, poly-D,L-lactic acid-para-dioxanone-polyethylene glycol block copolymer (PLA-DX-PEG), to serve as a biocompatible, biodegradable polymer for recombinant human (rh) BMP-2 delivery systems. In animal experiments, new bone was efficiently formed and a large bone defect was repaired using PLA-DX-PEG/rhBMP-2 composites. In addition, this new polymer could be used as an injectable delivery system for rhBMP-2. The rhBMP-2/PLA-DX-PEG composites also could be combined with other materials such as hydroxyapatite or titanium. This new synthetic polymer might be used for rhBMP-2 delivery in various clinical situations involving repair of bone, leading to great changes in orthopedic treatment.
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Keywords: Bone formation; Bone repair; Fracture; Bone defect; Recombinant human bone morphogenetic protein-2; Tissue engineering

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1. Introduction

The regeneration potential of human bone appears to be limited, given that repair of large bone defects such as those associated with comminuted fractures or bone tumor resection usually remains unrepaired [1]. Such cases have been treated routinely with autogeneic or allogeneic bone grafting. Major problems associated with autogeneic grafting include limited anatomic sources of donor bone and risk of morbidity from the additional surgery for procurement of the graft. In allogeneic bone grafting, major concerns are potential risks of transmission of disease, immunologic reaction of the host, poor osteogenic capacity of the transplanted bone, and high costs associated with a bone banking system [2–4]. Current examination of alternatives to grafting techniques suggests three possible new approaches to inducing new bone formation: implantation of certain cytokines such as bone morphogenetic proteins (BMP) in combination with appropriate delivery systems at the target site [5–7]; transduction of genes encoding cytokines with osteogenic capacity into cells at repair sites [8,9]; and transplantation of cultured osteogenic cells derived from host bone marrow [10–13]. In our estimation, the second approach represents the next major advance, while the third requires considerable additional resources and time to procure and culture cells. The first strategy appears to show the most practical promise for the near future. Appropriate delivery systems are essential to this technique. In this review, we outline the development of new delivery systems for BMP and preclinical animal experiments concerning bone tissue regeneration that suggest clinical applications.

2. Bone morphogenetic proteins (BMP) and delivery systems

2.1. BMP

BMP induce new bone formation by directing mesenchymal stem cells toward chondroblastic and osteoblastic differentiation, and causing them to proliferate in vivo. BMP expression has been confirmed to occur at the initial stage of the fracture healing process, and to participate in a cascade regulating bone repair processes. Also, new bone can be induced to form heterotopically, such as when the BMP are implanted in muscle in animal models using appropriate delivery systems. These observations suggest that BMP could be applied clinically to promotion of repair of bone.

BMP were first characterized in 1965 by Urist as a biologically active molecule inducing new ectopic bone formation from decalcified bone matrix in vivo [14]. A cDNA encoding BMP was cloned by Wozney

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

$$\underbrace{\text{H-(-O-CH(CH}_3\text{)-CO-)}_m\text{-O-}}_{\text{PLA segment}}\underbrace{\text{(-CH}_2\text{-CH}_2\text{-O-)}_n\text{-}}_{\text{PEG segment}}\underbrace{\text{(-OC-CH(CH}_3\text{)-O-)}_o\text{-H}}_{\text{PLA segment}}$$

PLA-DX-PEG

$$\underbrace{\text{H-(-O-CH(CH}_3\text{)-CO-)}_m\text{-(-O-CH}_2\text{-CH}_2\text{-O-CH}_2\text{-CO-)}_n}_{\text{PLA-DX random polymer segment}}\text{-O-}\underbrace{\text{(-CH}_2\text{-CH}_2\text{-O-)}_o\text{--}}_{\text{PEG segment}}$$

$$\underbrace{\text{--(-OC-CH}_2\text{-O-CH}_2\text{-CH}_2\text{-O-)}_p\text{-(-OC-CH(CH}_3\text{)-O-)}_q\text{-H}}_{\text{PLA-DX random polymer segment}}$$

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.

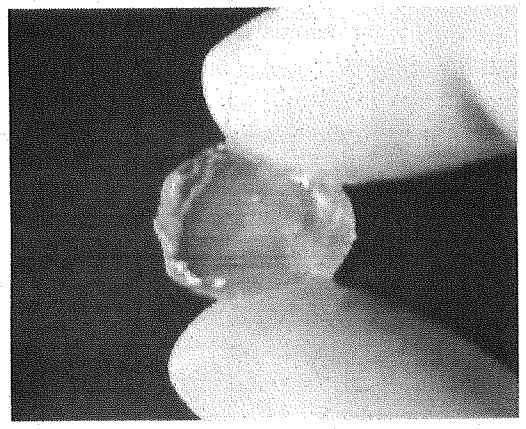

Fig. 2. Appearance of PLA-DX-PEG (reprinted from [51], with permission from Marcel Dekker Inc.) requires copyright permission since we previously published in Tissue engineering and novel delivery systems [51]. At room temperature, PLA-DX-PEG with a molecular weight of 9500 is a firm gel, which can be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

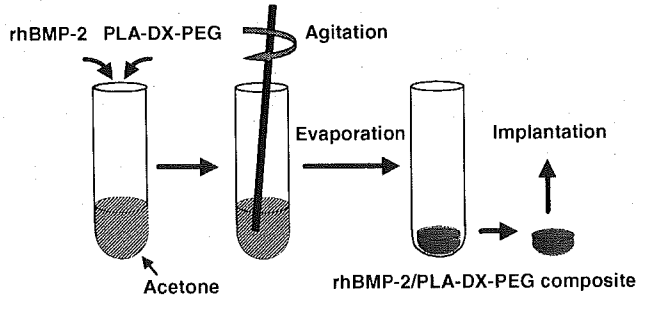

Fig. 3. Methods for combining PLA-DX-PEG polymer with rhBMP-2. PLA-DX-PEG was dissolved in organic solvent, and rhBMP-2 solution was mixed in it. Evaporation with a centrifuge evaporator removed the solvent, so an rhBMP-2/PLA-DX-PEG composite implant was obtained.

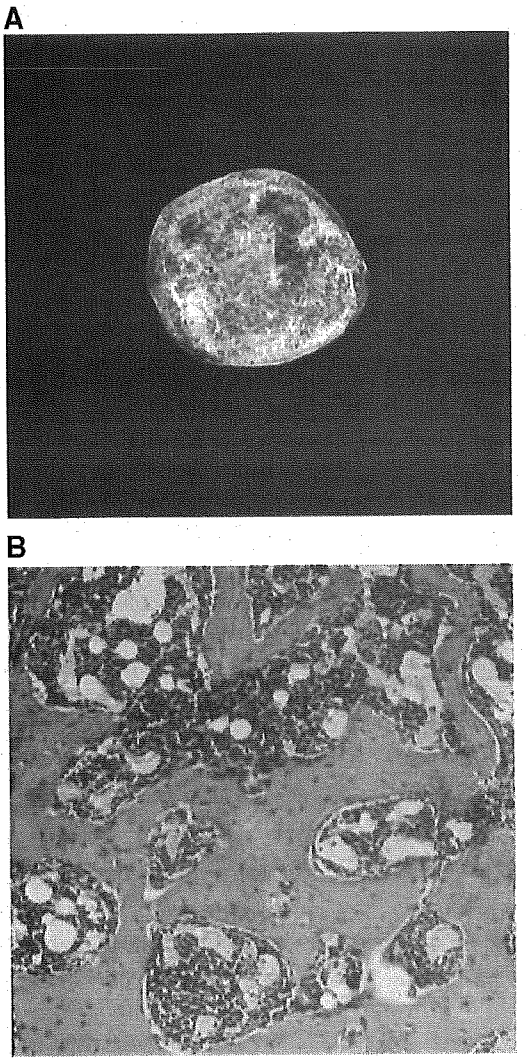

Fig. 4. Ectopic bone formation induced by the rhBMP-2/PLA-DX-PEG composite (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. The composite was placed in the back muscle of mouse, which was harvested 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

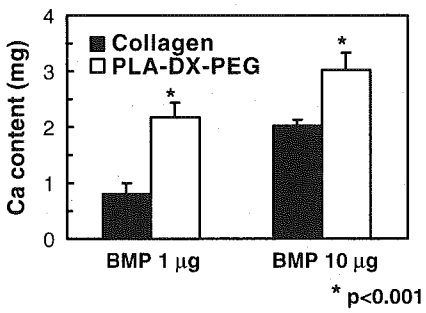

Fig. 5. Comparison of calcium content of new bone obtained using an PLA-DX-PEG system and a collagen system (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. The calcium content of new bone obtained 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

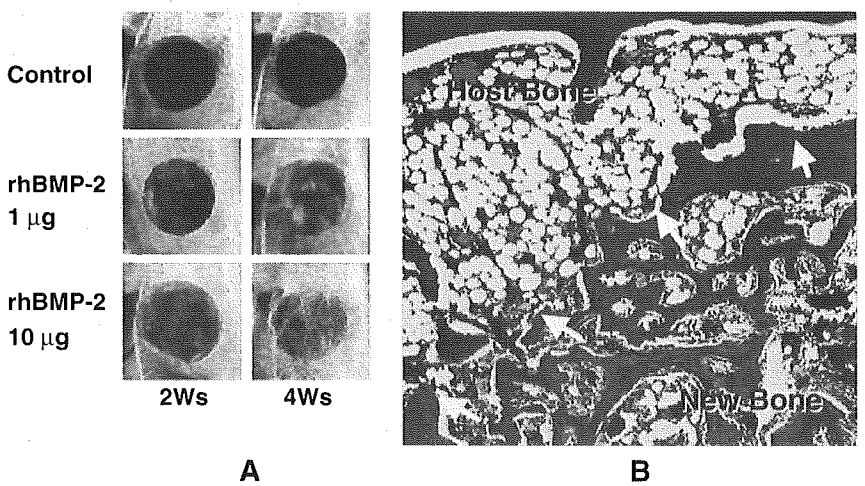

Fig. 6. Repair of a bone defect using PLA-DX-PEG as a delivery system for rhBMP-2 (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. A cylindrical defect 4 mm in diameter was created in the ilium of 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.

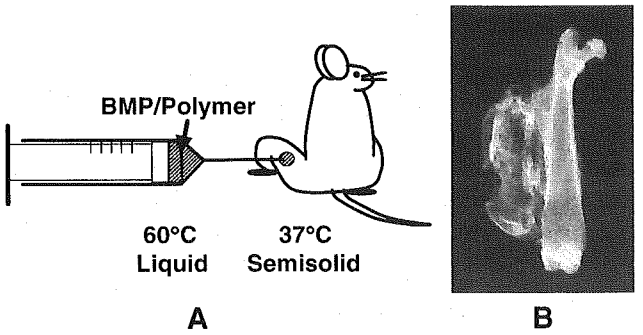

Fig. 7. Injectable polymeric delivery system for rhBMP-2 (reprinted from [56], with permission from Elsevier) requires copyright permission since we previously published in Bone [56]. (A) When heated to 60 °C, the rhBMP-2/PLA-DX-PEG composite can be injected percutaneously, avoiding need for surgical 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

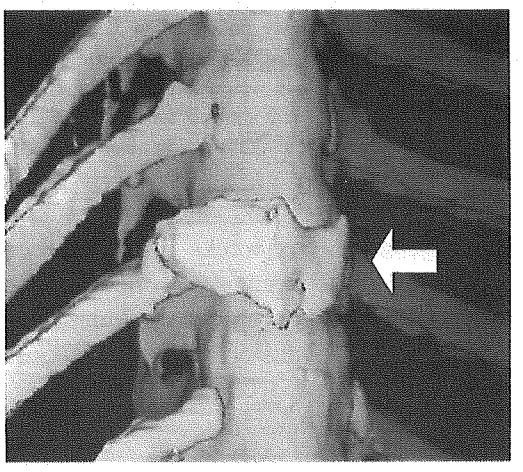

Fig. 8. Spinal fusion by injection of the rhBMP-2/PLA-DX-PEG composite (reprinted from [58], with permission from Lippincott Williams and Wilkins) requires copyright permission since we previously published in J. Spinal Disord. [58]. PLA-DX-PEG with rhBMP-2 was injected into the anterior longitudinal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

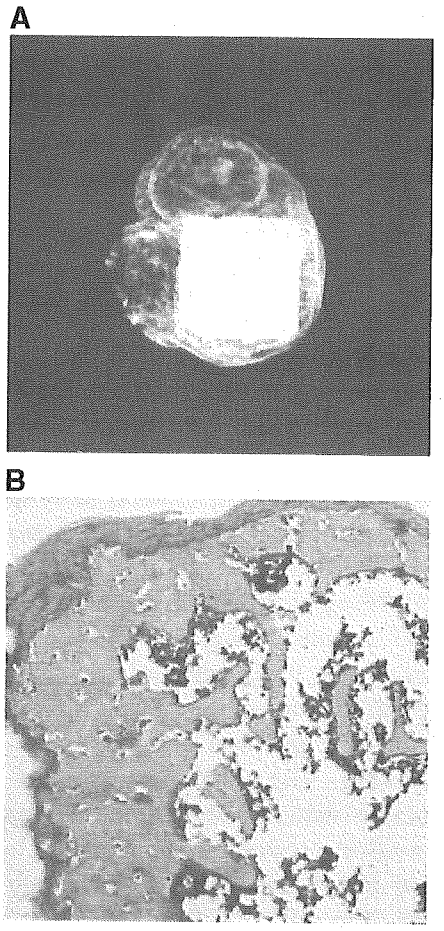

Fig. 9. Ectopic bone formation by hydroxyapatite (HA) with the rhBMP-2/PLA-DX-PEG (reprinted from [54], with permission from The Journal of Bone and Joint Surgery, Inc.) requires copyright permission since we previously published in J. Bone Joint Surg. Am. [54]. The rhBMP-2/PLA-DX-PEG composite was placed 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

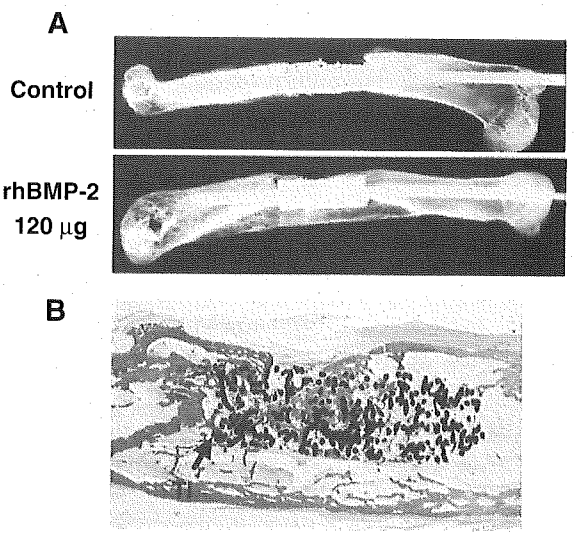

Fig. 10. Repair of a bone defect with a titanium syringe implant with the rhBMP-2/PLA-DX-PEG composite (reprinted from [59], with permission from 6 Wiley) requires copyright permission since we previously published in J. Biomed. Mater. Res. [59]. A bone defect of 1.5 cm was created in the humerus of 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.

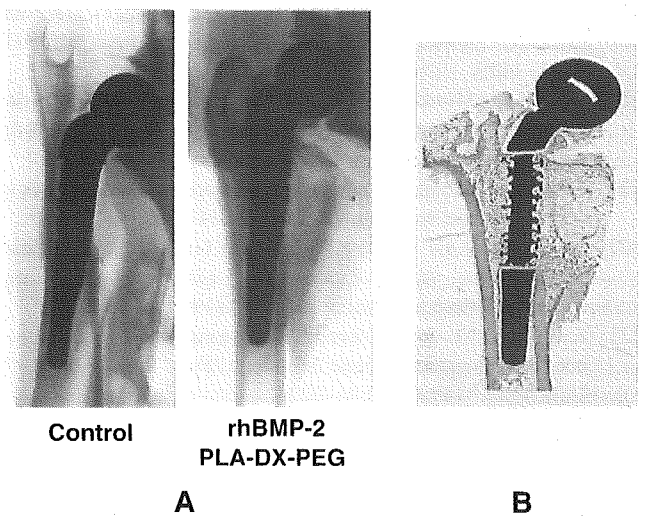

Fig. 11. Repair of a periprosthetic bone defect using PLA-DX-PEG/rhBMP-2 composite adherent to the prosthesis (reprinted from [60], with permission from Elsevier) requires copyright permission since we previously published in Biomaterials [60]. (A) Twelve weeks after implantation, the implant with rhBMP-2/PLA-DX-PEG 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.

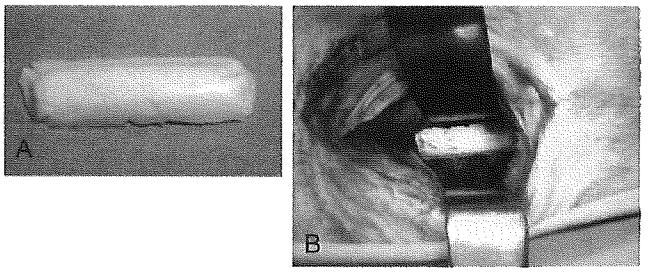

Figure 2. **A**, Prepared β-TCP dough implant. **B**, β-TCP dough was placed on the L5–L6 intertransverse region.

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-

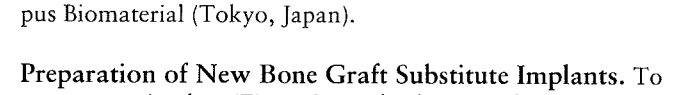

Figure 1. Structural formula of PLA-DX-PEG. The subscripts m, n, o, p, q represent variable number of units.

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.