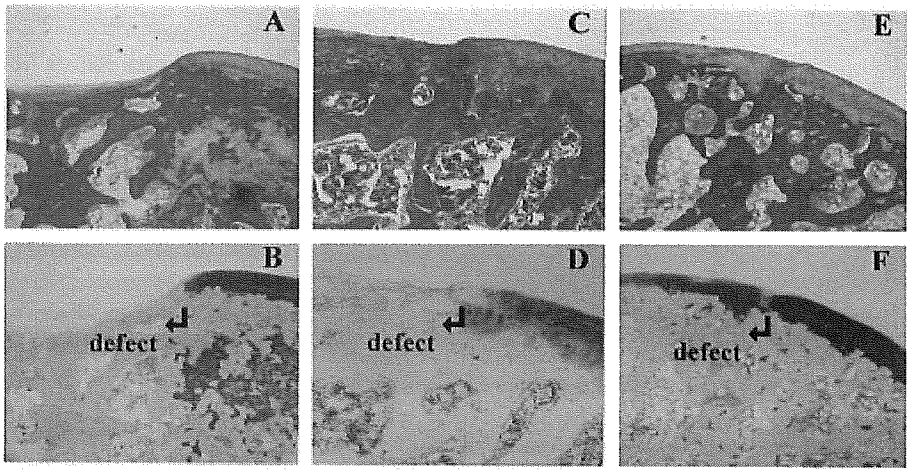

Figure 5. Osteochondral defects of a rat knee repaired with tissue pellets generated in diffusion chambers 24 weeks after transplantation. **A** and **B,** Defect with no implant. **C** and **D,** Defect implanted with tissue pellet generated in the chamber of group B0 (without recombinant human bone morphogenetic protein 2 [rHuBMP-2]). **E** and **F,** Defect implanted with tissue pellet generated in the chamber of group B10 (10 μg rHuBMP-2). (Stained with hematoxylin and eosin in **A, C,** and **E,** with toluidine blue in **B, D,** and **F**; original magnification × 40.)

obvious border with the surrounding normal articular cartilage (Figures 5E and F). The defects were filled with a layer of cartilage exhibiting subchondral cancellous bone connecting to the original subchondral bone. Although the architecture of the repaired articular cartilage was similar to that of normal cartilage with regard to cell arrangement, differences were noted. A tidemark was visible at the base of the cartilage layer adjacent to the subchondral bone, and the thickness of the regenerated cartilage was slightly less than that of the neighboring normal articular cartilage.

In contrast, the defects transplanted with tissue mass from group B0 were partially repaired, with a depressed surface visible at the defect site (Figures 5C and D). Histologic assessment of the defects that received either the tissue from group B0 or no implant revealed a small amount of fibrocartilage, with slightly positive metachromatic staining at the periphery of the defects and dominant fibrous tissue in the defect space.

Upon histologic evaluation of the knee cartilage after repair, the average histologic score (Wakitani's score) was 4.25 for group B10, 11.67 for group B0, and 14.00 for the defect-only group. The score for group B10 was significantly better than that for group B0 (P = 0.032) and the defect-only group (P = 0.002).

DISCUSSION

The experimental data presented herein indicate the capacity of rHuBMP-2 to induce the differentiation of young muscle-derived mesenchymal cells into chondrocytes within diffusion chambers in in vivo conditions. The resultant heterotopic cartilage formation represents a significant volume of induced tissue mass derived from these cells.

In order to induce the cartilage tissue, the diffusion chamber system was essential. When vascular invasion into the chamber occurred as a result of membrane seal failure, new bone with hematopoietic marrow was seen in the chambers harvested at 5 weeks after transplantation. Budenz and Bernard have reported similar findings (14). This bone was likely formed through the process of endochondral ossification, as deduced from classic reports describing the actions of BMP (15) and from comparison with the process of direct ossification (16,17). In the process of BMP-induced endochondral bone formation, cartilage is formed in the early phase of the bone-forming process. The cartilage tissue is then absorbed by invading vascular connective tissue and replaced by newly formed bone, as seen in embryonic osteogenesis (18) and in callus in fracture repair (19). During the process of ectopic bone formation elicited by

BMP, Tsuyama et al (20) found that the induced bone marrow cells are not the progeny of undifferentiated mesenchymal cells in situ, but rather arise from hematopoietic stem cells circulating in the peripheral blood. This conclusion was based on studies of chimeric mice and bone marrow transplantation (20).

In this diffusion chamber system, the cells within the chambers were able to survive by diffusion of tissue fluid from host animals, but vascular invasion was blocked by the filter membranes. As a result, the BMP-induced bone-forming reaction was stopped at the stage of cartilage formation and pieces of cartilage for transplantation were obtained, although it took a period of 5–6 weeks to achieve this outcome. This is a longer timeframe than the time taken by collagen pellets with rHuBMP-2 to form ectopic bone. In the ectopic endochondral bone formation process, ossification starts at the border of the cartilage and surrounding tissue.

The results of the RT-PCR analysis of type II collagen and aggrecan revealed that muscle-derived mesenchymal cells differentiated into chondrocytes at 4 days after implantation. However, mature cartilage matrix synthesis started a few days later, since the expression of type IX collagen, which is essential for type II collagen to form cartilage matrix, was weak at 4 days and increased significantly by day 7. Type X collagen and type XI collagen were detected by RT-PCR either with or without rHuBMP-2 in these cells. Because type II collagen and aggrecan were not detected initially, we cannot be sure that chondrogenesis started at the 0 time point. Further work will be needed to map out the exact sequence of expression of these genes in this model. In the absence of rHuBMP-2, the cells expressed type II collagen, but the level was much less when compared with that in cells with rHuBMP-2. This might mean that slow chondrogenesis of muscle-derived mesenchymal cells might occur even in the absence of rHuBMP-2 in this condition.

To examine whether osteogenic differentiation of the muscle-derived mesenchymal cells occurred in this system, we detected Cbfa1/Runx2, which is an essential transcriptional factor for osteoblastic differentiation, by RT-PCR. The expression of Cbfa1/Runx2 was observed at 96 hours in chambers with rHuBMP-2 but not observed in the absence of rHuBMP-2, which means that osteogenic differentiation was initiated by rHuBMP-2. We could not detect the expression of MyoD1 nor were there any cells showing a myogenic phenotype either in the chambers or in the defects at any time point.

The diffusion-chamber–engineered cartilage mass was able to repair full-thickness cartilage defects. At 24 weeks after transplantation, the transplanted cartilage was incorporated and effectively repaired the cartilage defects. The superficial layer of the transplant facing the joint surface had histologic characteristics of articular cartilage, but the greater part beneath the cartilage layer was replaced by bone mass, which was connected to the original subchondral bone. This morphologic condition suggests that part of the transplanted cartilage mass appeared to have features of preossifying cartilage and was in the process of remodeling. This adaptation to the surrounding environment also has been observed in an experiment involving cell transplantation to correct an osteochondral defect (13). When the cartilage plugs that were made in diffusion chambers were implanted into the osteochondral defects, they were replaced by bone from the bone marrow side, but the surface area that was in contact with the joint space remained as cartilage. We believe that the implanted chondrocytes remained at the surface of the defect, although there are no data to support this conclusion.

Adachi et al (21) reported that allogeneic muscle-derived cells embedded in collagen gels are useful for repair of full-thickness articular cartilage, both as a gene delivery vehicle and a cell source for tissue repair. They transduced rabbit allogeneic muscle-derived cells with the β-galactosidase gene (LacZ) and transplanted the cells into the osteochondral defects in the patellar groove in rabbit knees. They reported that the LacZ-positive cells were found in the defect only up to 4 weeks after transplantation. Further studies will be required to more completely understand the biochemical and morphologic processes that underpin the restorative actions of these cell and tissue transplants.

Although the generation of the new cartilage mass and repair of a cartilage defect with the engineered cartilage were shown to be successful in rats, there are some hurdles to be cleared before this approach can be applied in clinical practice. In this study, we used cells from the embryo, which were thought to be more primitive and to have greater capacity for differentiation. However, this represents a problem for clinical application, because of ethical and regulatory issues. Our technique could be applied to muscle-derived cells from the adult, and in this approach, we can use autologous cells. We are planning to apply this system to adult cells, such as bone marrow mesenchymal cells, adipocytes, and muscle-derived cells.

The less responsive nature of muscle-derived mesenchymal cells to rHuBMP-2 in large mammals, including humans, could also be an issue (22). Moreover, the optimal dose of BMP required for cartilage induc-

tion in humans must be determined. In order to solve these issues, further experimental studies in large animals will be essential.

The kinetics of BMP release from collagen is an important consideration. Sellers et al (23) reported that the mean residence time of rHuBMP-2 from a collagen sponge impregnated with 5 μg of rHuBMP-2 was 8 days, with an elimination half-life of 5.6 days. In addition, detectable amounts of rHuBMP-2 were present as long as 14 days after implantation.

In comparing the data from the present study with those reported by Sellers et al, there are differences in experimental details. Sellers et al implanted collagen with 5 μg of rHuBMP-2 into the osteochondral defect, which is likely to result in a rapid vascular invasion and much faster degradation. In the present study, collagen with 10 μg of rHuBMP-2 was placed into the chamber and implanted into subfascial pockets. The presence of the collagen in the chamber impeded invasion by the host cells. In addition, the preparation of a collagen gel and BMP-2 construct were different, and it would be reasonable to expect that the kinetics of BMP release would be influenced by these differences. It is also possible that the transplanted pellets might include BMP-2 at the time of implantation. Consequently, it would be the BMP-2, and not the cells in the pellet, that would drive the regeneration and the stability of repair cartilage. Further studies will be required to understand the kinetics of BMP release and the phenotypic stability of the transplanted cell population, which is believed to play a critical role in the outcome of tissue formation in vivo (24).

The present study has demonstrated another unique application of this approach, namely, the use of muscle-derived mesenchymal cells cultivated in an ex vivo system and differentiation of those cells into chondrogenic cells by rHuBMP-2 in diffusion chambers in an in vivo environment. Use of the muscle-derived mesenchymal cells together with rHuBMP-2 might be a reason for the successful generation of cartilage in this study, because these cells are known to have multilineage differentiation potential (21,25–27). While the present report provides evidence to support this approach for the successful treatment of articular cartilage defects, further studies will be needed to validate the technique for application in clinical practice.

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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.

Keywords: Bone formation; Bone repair; Fracture; Bone defect; Recombinant human bone morphogenetic protein-2; Tissue engineering

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Contents

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

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

PLA-DX-PEG

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

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

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.

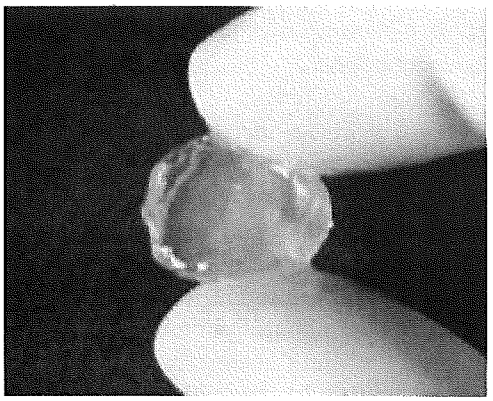

Fig. 2. Appearance of PLA-DX-PEG (reprinted from [51], with permission from Marcel Dekker Inc.) requires copyright permission since we previously published in Tissue engineering and novel delivery systems [51]. At room temperature, PLA-DX-PEG with a molecular weight of 9500 is a firm gel, which can be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

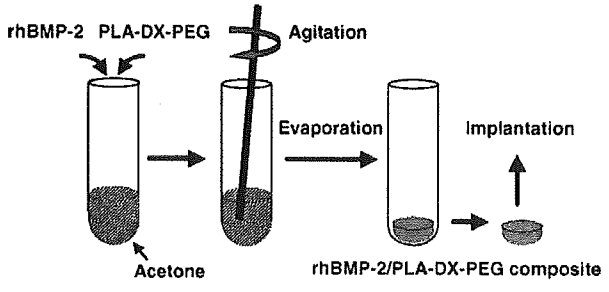

Fig. 3. Methods for combining PLA-DX-PEG polymer with rhBMP-2. PLA-DX-PEG was dissolved in organic solvent, and rhBMP-2 solution was mixed in it. Evaporation with a centrifuge evaporator removed the solvent, so an rhBMP-2/PLA-DX-PEG composite implant was obtained.

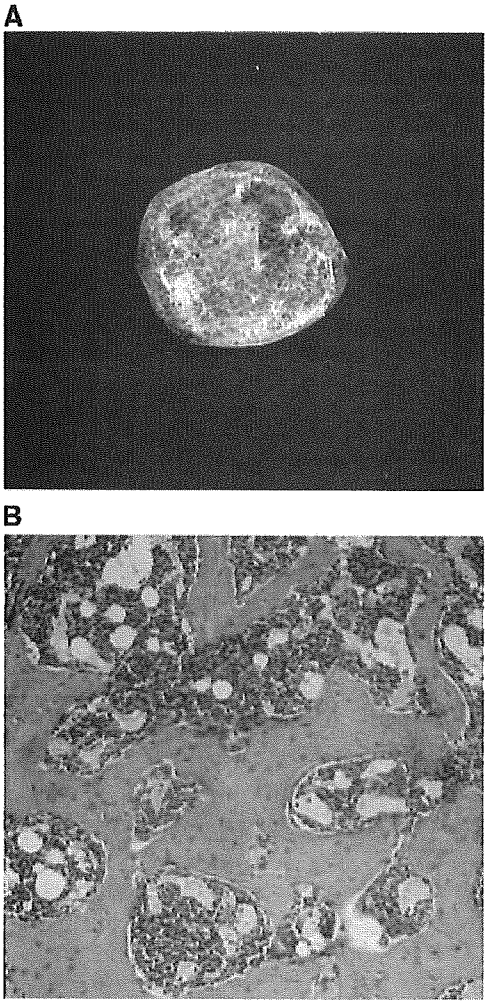

Fig. 4. Ectopic bone formation induced by the rhBMP-2/PLA-DX-PEG composite (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. The composite was placed in the back muscle of mouse, which was harvested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

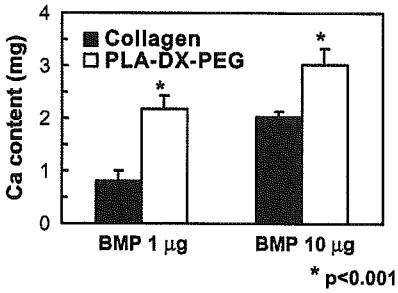

Fig. 5. Comparison of calcium content of new bone obtained using an PLA-DX-PEG system and a collagen system (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. The calcium content of new bone obtained 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 post-operatively. At the host–defect interface, new bone had formed adjacent to the host bone (Fig. 6). These

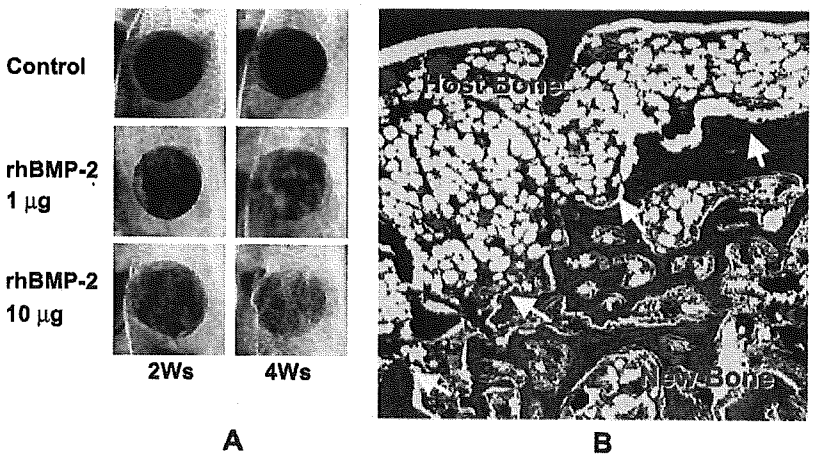

Fig. 6. Repair of a bone defect using PLA-DX-PEG as a delivery system for rhBMP-2 (reprinted from [55], with permission from Nature Publishing Group) requires copyright permission since we previously published in Nat. Biotechnol. [55]. A cylindrical defect 4 mm in diameter was created in the ilium of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

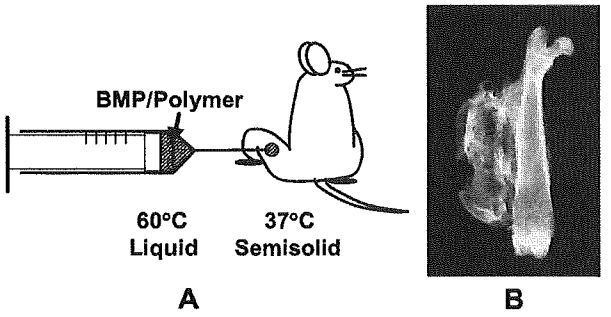

Fig. 7. Injectable polymeric delivery system for rhBMP-2 (reprinted from [56], with permission from Elsevier) requires copyright permission since we previously published in Bone [56]. (A) When heated to 60 °C, the rhBMP-2/PLA-DX-PEG composite can be injected percutaneously, avoiding need for surgical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

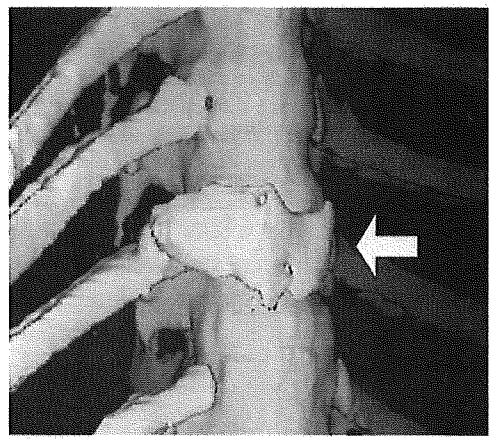

Fig. 8. Spinal fusion by injection of the rhBMP-2/PLA-DX-PEG composite (reprinted from [58], with permission from Lippincott Williams and Wilkins) requires copyright permission since we previously published in J. Spinal Disord. [58]. PLA-DX-PEG with rhBMP-2 was injected into the anterior longitudinal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

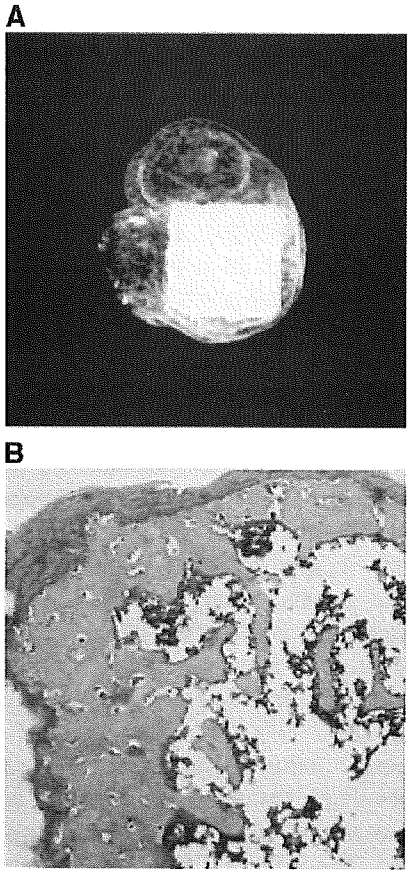

Fig. 9. Ectopic bone formation by hydroxyapatite (HA) with the rhBMP-2/PLA-DX-PEG (reprinted from [54], with permission from The Journal of Bone and Joint Surgery, Inc.) requires copyright permission since we previously published in J. Bone Joint Surg. Am. [54]. The rhBMP-2/PLA-DX-PEG composite was placed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

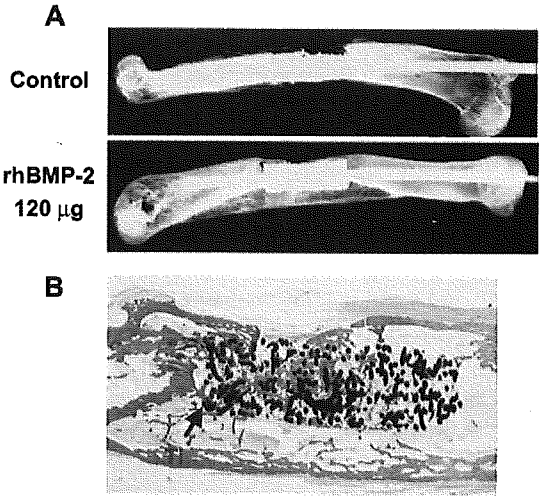

Fig. 10. Repair of a bone defect with a titanium syringe implant with the rhBMP-2/PLA-DX-PEG composite (reprinted from [59], with permission from 6 Wiley) requires copyright permission since we previously published in J. Biomed. Mater. Res. [59]. A bone defect of 1.5 cm was created in the humerus of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

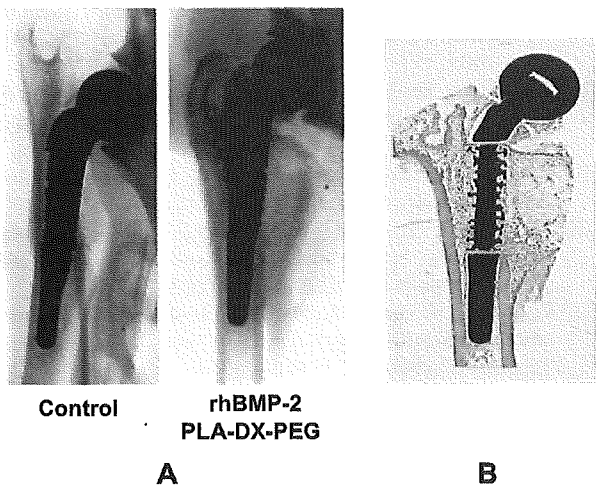

Fig. 11. Repair of a periprosthetic bone defect using PLA-DX-PEG/rhBMP-2 composite adherent to the prosthesis (reprinted from [60], with permission from Elsevier) requires copyright permission since we previously published in Biomaterials [60]. (A) Twelve weeks after implantation, the implant with rhBMP-2/PLA-DX-PEG 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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EBMに基づく骨粗鬆症の薬物療法：ビスフォスフォネート製剤の使い方

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- 骨粗鬆症の薬物療法のゴールは骨折予防である．
- 現在わが国で骨粗鬆症治療薬として認可されているビスフォスフォネートは，エチドロネート，アレンドロネート，リゼドロネートの3剤である．
- 特に後2剤は大規模試験によって骨折予防効果が示され，骨粗鬆症治療のガイドラインでも第1選択薬として推奨されている．
- これら3剤の骨折予防効果のEBMを，椎体骨折と非椎体骨折に区別して概説する．
- EBMに基づいた薬剤の使用が推奨される．

Key Words　骨粗鬆症，ビスフォスフォネート，根拠に基づく医療（EBM），骨折予防

はじめに

現在わが国で骨粗鬆症治療薬として認可されているビスフォスフォネート（BP）は，エチドロネート（EHDP），アレンドロネート（ALN），リゼドロネート（NE）の3剤である．骨粗鬆症の薬物療法のゴールは骨折予防であるが，特に後2剤は大規模試験によって骨折予防効果が示され，骨粗鬆症治療のガイドラインでも第1選択薬first line drugとして推奨されている[1~5]．これら3剤の骨折予防効果のEBMを，椎体骨折（表1）と非椎体骨折（表2）に区別して概説する．

□ ビスフォスフォネート(BP)の骨吸収抑制作用

BPは主として破骨細胞もしくはその前駆細胞に作用して，骨吸収能を阻害する[6]．その作用機序としては，破骨細胞への分化抑制，破骨細胞への細胞毒性・細胞内器官破壊，破骨細胞の骨への接着阻害などが示されている．骨吸収抑制作用の強さによって，一般に第1~3世代に分類されている．第1世代は石灰化抑制と骨吸収抑制の両作用をもつ薬剤で，EHDPがその代表である．両作用の現れる用量差を大きくしたBPが第2世代で，ALNやパミドロネートがある．さらに，実験動物に致死量に近い量を投与しても石灰化抑制が見られず，骨吸収抑制が強いBPが第3世代と呼ばれ，NEがその代表である．ラットの脛骨近位で測定した骨吸収抑制作用はおよそ，EHDP：ALN：NE＝1：1000：5000である．

□ エチドロネート（EHDP）の骨折予防効果

1．海外でのEBM

Stromらは，閉経後骨粗鬆症患者66人で，EHDPの無作為対照試験（RCT）を約3年間（150週）行った[7]．EHDPの新規椎体骨折発生抑制は58%であったが有意でなく，経過観察期間を60~150週に絞った解析で89%と有意な抑制効果を認めた．Wattsらは[8]，閉経後1年以上で1~4個の既存椎体骨折がある429人を対象にRCTを2年間行い，56%と有意な椎体骨折発生抑制率を得た．

2．わが国でのEBM

藤田らが閉経後および老人性骨粗鬆症患者429人を対象に1年間のRCTを行い，有意な椎体骨折発生抑制効果を得た[9]．

3．メタ解析

CranneyらによるRCTのメタ解析によれば，37%の椎体骨折発生抑制効果があるが，非椎体骨折発生抑制効果はない[10,11]．

□ アレンドロネート（ALN）の骨折予防効果

1．海外でのEBM

Libermanらは[12]，骨量減少と骨粗鬆症女性

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表1　ビスフォスフォネート（BP）の無作為プラセボ対照試験での椎体骨折（VF）発生抑制効果

BP	報告 試験略称　年	対　象	経過 観察	VF 発生 抑制	骨密度変化 部位
EHDP	Storm et al[7] 1990	閉経後骨粗鬆症 66 人	150 週	60-150 週で 89%	5.3%　LS
EHDP	Watts et al[8] 1990	閉経後 1 年以上 VF 1-4 個ある 429 人	2 年	56%	4.2%, 5.2%　LS
EHDP	藤田ら[9] 1993	閉経後・老人性 骨粗鬆症 268 人	1 年	有意	2.4%（200 mg） 3.4%（400 mg） LS
EHDP	Cranney et al[10] 2001	13 試験のメタ解析 1267 人	1 年以上	37%	4.06%　LS 2.35%　FN 0.97%　TB
EHDP	Cranney et al[11] 2002	9 試験のメタ解析 1076 人	1 年以上	37%	
ALN	Liberman et al[12] 1995	骨量減少・骨粗鬆症 女性 994 人 （既存 VF ありが 20%）	3 年	48%	8.8%　LS 5.9%　FN 2.5%　TB
ALN	Black et al[13] FIT 1996	既存 VF がある骨粗鬆症 女性 2027 人	2.9 年	47% 55%* 90%**	8.0%　LS 3.6%　FN 3.0%　TH
ALN	Cummings et al[14] FIT 1998	既存 VF がない骨量減少 骨粗鬆症女性 4432 人	4.2 年	44%	8.3%　LS 3.8%　FN 3.4%　TH
ALN	Black et al[15] FIT 2000	骨粗鬆症女性 3658 人	3-4 年	48% 45%* 87%**	
ALN	Kushida et al[25]	既存 VF 1-4 個がある骨粗鬆症女性 170 人（アルファカルシドール対照）	3 年	59%	9.2%　LS
ALN	Cranney et al[11]	8 試験のメタ解析 9360 人		48%	
NE	Harris et al[27] VERT-NA 1999	VF がある閉経後女性 939 人	3 年	41%	5.4%　LS 1.6%　FN 3.3%　TR
NE	Reginster et al[28] VERT-MN 2000	VF がある閉経後女性 816 人	3 年	49%	7.0%　LS 2.0%　FN
NE	Heaney et al[29]	既存 VF がない骨粗鬆症 女性 640 人	1.5-3 年	75%	5.3%（3 年）LS 2.5%（3 年）FN
NE	Kushida et al[36]	既存 VF 1-4 個がある骨粗鬆症患者 547 人（EHDP 対照）	3 年	非劣性	
NE	Cranney et al[11] 2002	5 試験のメタ解析 2604 人		36%	

*症状のある臨床骨折抑制率

**多発骨折抑制率

骨折発生抑制の%値は有意な抑制率（NS：not significant）.

（部位）LS：lumbar spine, FN：femoral neck, TR：trochanter, TH：total hip, Hip-fx：hip fracture, TB：total body

表2　ビスフォスフォネート（BP）の無作為プラセボ対照試験での非椎体骨折（NVF）発生抑制効果

BP	報告 試験略称　年	対　象	経過 観察	NVF 発生 抑制	骨密度変化 部位
EHDP	Cranney et al[10] 2001	13 試験のメタ解析 1267 人	1 年以上	NS	4.06%　LS 2.35%　FN 0.97%　TB
EHDP	Cranney et al[11] 2002	7 試験のメタ解析 867 人	1 年以上	NS	
ALN	Liberman et al[12] 1995	骨量減少・骨粗鬆症 女性 994 人 （既存 VF ありが 20%）	3 年	NS	8.8%　LS 5.9%　FN 2.5%　TB
ALN	Black et al[13] FIT 1996	既存 VF がある骨粗鬆症 女性 2027 人	2.9 年	51% Hip-fx 48% Wrt-fx	8.0%　LS 3.6%　FN 3.0%　TH
ALN	Cummings et al[14] FIT 1998	既存 VF がない骨粗鬆症 女性 4432 人	4.2 年	56% Hip-fx	4.6%　FN
ALN	Pols et al[23] FOSIT 1999	閉経後骨粗鬆症 1908 人	1 年	47% NVF	5.0%　LS 2.3%　FN 4.1%　TR 3.1%　TH
ALN	Black et al[15] FIT 2000	骨粗鬆症女性 3658 人	3-4 年	27% NVF 53% Hip-fx 30% Wrt-fx	
ALN	Cranney et al[11] 2002	8 試験のメタ解析（5 mg） 8603 人 6 試験のメタ解析（10-40 mg） 3723 人		NS 49% NVF	
ALN	Papapoulos et al[26]	6 試験のメタ解析（5-20 mg） T≦−2.0 SD か既存 VF あり 9023 人 T≦−2.5 SD か既存 VF あり 6804 人	1-4.5 年	 45% Hip-fx 55% Hip-fx	
NE	Harris et al[27] VERT-NA 1999	VF がある閉経後女性 939 人	3 年	39% NVF	5.4%　LS 1.6%　FN 3.3%　TR
NE	McClung et al[30] HIP, 2000	70-79 歳骨粗鬆症女性 5445 人 80-歳で Hip fx リスク ある女性 3886 人	3 年 3 年	40%　Hip-fx NS Hip-fx	
NE	Cranney et al[11] 2002	7 試験のメタ解析 12958 人		27% NVF	

*症状のある臨床骨折抑制率

**多発骨折抑制率

骨折発生抑制の%値は有意な抑制率（NS：not significant）.

（部位）LS：lumbar spine, FN：femoral neck, TR：trochanter, TH：total hip, Hip-fx：hip fracture, Wrt-fx：wrist fracture, TB：total body